AF/1621
RFW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

SCOTT MILLER		Confirmation No.:	
Serial No.:	**09/776,936**	Examiner:	**KUMAR, SHAILENDRA**
Filed:	**12/22/98**	Group Art Unit:	**1621**

Title: **INHIBITION OF RAF KINASE USING SYMMETRICAL AND UNSYMMETRICAL SUBSTITUTED DIPHENYL UREAS**

REPLY BRIEF

MAIL STOP APPEAL BRIEF – PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Examiner's Answer mailed January 3, 2006, herewith is Appellant's Reply Brief.

The Examiner still has not presented any evidence or adequate reason for maintaining the rejection under 35 U.S.C. § 112, first paragraph of claims 15-19 or for maintaining the rejection under 35 USC § 103. The Reply Brief is presented in response to the following new points of argument raised in the Examiner's Answer:

1) In addressing the factors under In re Wands, 8 USPQ 2d 1400 (1988), as it relates to the rejection of claims 15-19 under 35 USC 112, first paragraph, applicants disagree with the characterization of the state of prior art on page 4, lines 4-8 of the Examiner's Answer, which reads:

> **The state of the prior art:** while the state of the art is relatively high with regard to the treatment of cancerous cell growth, the state of the art with regard to a single agent for treating cancer broadly is underdeveloped. In particular, there is no known anticancer agent, which is effective against cancer such as pancreatic, lung and colon, thyroid or bladder for that matter.

No evidence or publication has been cited to support these conclusions. In fact many anticancer agents are used for the treatment of more than one cancer. For example, paclitaxel is used to treat ovarian cancer, head and neck cancer, non-small cell lung cancer, small cell lung cancer, bladder cancer and advanced breast cancer. Vinorelbine has been given for the treatment of breast cancer, non-small cell lung cancer, ovarian cancer, and Hodgkin's disease. Cisplatin is given to treat testicular, bladder, lung, and ovarian cancers, head and neck cancer, cervical carcinoma and neurologic cancers. See http://www.nlm.nih.gov/medlineplus/druginformation.html. While an anticancer agent has not been approved for the treatment of pancreatic, lung and colon, thyroid or bladder, this bears no relevance as to whether claims 15-19 meet the requirements of 35 USC § 112.

Applicants also submit the state of the art with respect to raf kinase inhibitors is more relevant to the issues under 35 USC § 112, some of which is discussed in the specification and the references of record.

By the early 1990s the raf oncogene had been discovered, its role in oncogenesis had been elucidated and the role of raf in the ras-raf signaling cascade had been determined. See Reference of record, Avruch et al. "Raf meets Ras: completing the framework of a signal transduction pathway, TIBS 19; July 1994; pp 279-281.

Storm, S. M.; Brennscheidt, U.; Sithanandam, G.; Rapp, U. R., raf oncogenes in carcinogenesis. Critical reviews in oncogenesis 1990, 2, (1), 1-8, reports that there is a role of raf oncogenes in human tumors, e.g., various leukemias, larynx carcinoma, breast carcinoma, lung carcinoma, and renal cell carcinoma.

This led to disclosures of antisense approaches to inhibition of raf signaling. See Reference of record, Monia, B. P.; Johnston, J. F.; Geiger, T.; Muller, M.; Fabbro, D., Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-raf kinase. *Nature Medicine* **1996,** 2, (6), 668-675.

Naumann, U. H., Angelika; Flory, Egbert; Rapp, Ulf R., Raf protein serine/threonine kinases. Protein Phosphorylation 1996, 203-236, teach that there are various lines of evidence pointing to a role of raf in malignant transformation, and teaches that transforming versions of c-raf-1 have been detected in fibroblasts following transfection with DNA from various tumor cells including a human glioblastoma cell line, primary stomach cancer cells,

chemically induced rat hepatocarcinoma cells, cells derived from renal and breast carcinoma as well as lung carcinoid.

This work was followed by approaches dependent on inhibition of raf signaling and by the mid-late 1990's a number of research groups disclosed assays for measuring the ability of compounds to inhibit raf activity. See for example:

Heimbrook, D. C.; Oliff, A. I.; Stirdivant, S. M. Preparation of imidazole derivatives and imidazole-contg. peptide analogs and a method of treating cancer. WO 199736587, 19970331, 1997;

Naumann, U.; Eisenmann-Tappe, I.; Rapp, U. R., The role of Raf kinases in development and growth of tumors. *Recent Results in Cancer Research* **1997,** 143, (Risk and Progression Factors in Carcinogenesis), 237-244; and

Alessi, D. R. C., Philip; Ashworth, Alan; Cowley, Sally; Leevers, Sally J.; Marshall, Christopher J. , Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. *Methods in Enzymology* **1995,** 255, 279-90.

Therefore, at the time of applicant's invention, assaying for raf inhibition was an accepted means of identifying active compounds and raf inhibition was correlated with treating various forms of cancer.

2) The Examiner's Answer alleges on page 7 that simply IC_{50} data is provided and that applicants did not provide an "explanation that would suggest as to how these data are related to the treatment of various cancers." Applicants' assay and the data obtained are consistent with the assays and results others in the art used to identify compounds for treating cancers mediated by raf kinase. See, e.g., WO 97/36587. The data are clearly adequate to enable one of ordinary skill in the art to perform the claimed methods.

3) Applicants also disagree with the Examiner's characterization of another Wands factor, the predictability or lack thereof in the art. The Examiner's Answer does not address the predictability in the art but instead focuses on whether practicing the claimed invention is "unpredictable," see page 4, lines 9-12. Some of the prior art references mentioned above teach or suggest the use of raf kinase inhibitors in treating various cancers is predictable. For example, Monia, reference of record, states within the abstract:

"Oligodeoxynucleotide treatment resulted in potent antiproliferative effects in cell culture and potent anti-tumor effects in vivo against a variety of tumor types that were highly consistent with an antisense mechanism of action for these compounds. These strongly suggest that antisense inhibitors targeted against c-raf-1 kinase may be of considerable value as antineoplastic agents that display activity against a wide spectrum of tumor types." (Emphasis added.)

WO 98/22103, reference of record, cites six references on pages 1 and 2 which are said to provide "evidence that inhibitors of raf will result in anti-tumor activity," and taken together these references indicate that:

"raf is both a direct and major effector of ras function and as such inhibition of the kinase activity of raf is expected to have antitumour activity in at least a proportion of human tumors. Specific cancers of interest include:

carcinoma, including that of the bladder, breast, colon, kidney, liver, lung, ovary, pancreas, stomach, cervix, thyroid, and skin;

hematopoietic tumors of lymphoid lineage, including lymphocytic leukemia, B-cell lymphoma, and Burketts lymphoma;

hematopoietic tumors of myeloid lineage including acute and chronic myelogenous leukemias and promyelocytic leukemia;

tumors of mesenchymal origin, including fibersarcoma and rhabdomya sarcoma; and other tumors, including melanoma, seminoma, tetratocarcinoma; neuroblastoma and glioma." (Emphasis added.)

WO 97/36587 states on page 1, "Since inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals, these studies suggest that the antagonism of Raf is an effective means by which to treat cancers in which Raf plays a role.

Examples of cancers where Raf is implicated through over expression include cancers of the brain, genitourinary tract, lymphatic system, stomach, larynx, and lung."

Based on the above teachings, effective treatment of cancers, where Raf plays a role, with a raf kinase inhibitor, is predictable.

4) Applicants disagree with the Examiner's statement on page 4, lines 13-15, that, "The guidance given by the specification as to how to treat the solid tumor is limited [to]

the discussion of the literature only." and on page 5, lines 6-8, that, "Applicants have failed to provide guidance and information to allow the skilled worker to ascertain which particular type of cancer the claimed anticancer agent is effective against." As discussed in greater detail in the Appeal brief, the specification provides guidance as to dosages (pages 17-18, and 75-76), methods of administration (pages 13 to 18), activity (page 74) and cancers to be treated (page 2). Guidance given by the specification is not limited to a discussion of the literature.

5) The Examiner is requiring that the application meet clinical standards as set by the FDA to satisfy the enablement requirement under 35 U.S.C. § 112, first paragraph, which is inconsistent with prevailing Federal Circuit case law. For example, in addressing the issues under 35 U.S.C. § 112, first paragraph, the Examiner's Answer states on page 4, lines 9-12, "The lack of significant guidance from the specification or prior art [SIC] with regard to the actual treatment [of] solid cancers in [a] human subject with the claimed compounds makes practicing the claimed invention unpredictable, " and at the bottom of page 4 to line 2 of page 5, the Examiner's Answer states, "There is not a single example provided which can point out to the treatment of the solid tumor."

The actual treatment of solid cancers with the claimed compounds is not necessary to satisfy the enablement requirement of 35 USC ' 112, first paragraph. In *Cross et al. v. Iizuka et al.*, 224 USPQ 739 (CA FC 1985), where the opinion focused more on practical utility, but also held that enablement was met, stated that

> *in vitro* results with respect to the particular pharmacological activity are generally predictive of *in vivo* test results, i.e., there is a reasonable correlation therebetween. Were this not so, the testing procedures of the pharmaceutical industry would not be as they are. Iizuka has not urged, and rightly so, that there is an invariable exact correlation between in vitro test results and in vivo test results. Rather, Iizuka's position is that successful in vitro testing for a particular pharmacological activity establishes a significant probability that in vivo testing for this particular pharmacological activity will be successful.

The court further held that it finds itself

> in agrement with the Board that, based upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed in vitro utility and an in vivo activity, and therefore a rigorous correlation is not necessary where the

disclosure of pharmacological activity is reasonable based upon the probative evidence.

6) The Examiner's Answer also alleges on page 6 that many times *in vitro* studies can hardly be extrapolated to the *in vivo* studies. This is merely a bare allegation without a single example, and especially no example relevant to the present invention. From the foregoing discussion in section 1), it is clear to one of ordinary skill in the art that at the time of the invention an *in vitro* showing of raf activity in vitro is correlated with the treatment of various cancers.

Applicants in the present case have demonstrated that the compounds of the invention possess the relevant *in vitro* activity, see page 74 of specification and provided guidance as to how to use this knowledge in the treatment of various kinds of cancers. In view of the foregoing, applicants provided an enabling disclosure.

For the convenience of the Examiner and Board, some the references discussed are attached.

With respect to other issues raised in the Examiner's Answer, e.g., section 103 issues, applicants stand by their statements in the Appeal Brief.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,

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Attorney Docket No.: **Bayer-6-P1**

Date: **March 3, 2006**

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CERTIFICATION OF MAILING

I hereby certify that this correspondence is being deposited with the U.S. Postal Services as First Class Mail in an envelope addressed to: Commissioner of Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on: March 3, 2006

Name: Csaba Henter

Signature: _____

MILLEN, WHITE, ZELANO & BRANIGAN, P.C.

4. Cells in each well are lysed in 1 ml of 1% (w/v) SDS, transferred to a 15-ml round-bottomed polypropylene snap-cap tube (Falcon, Oxnard, CA), the wells washed with 0.5 ml of PBSA, and the wash combined with the SDS cell lysate. Two milliliters of ice-cold 15% (w/v) trichloroacetic acid (TCA) is added to each tube, the contents vortexed, and macromolecules precipitated by incubation on ice for 10 min. The precipitate from each tube is collected on a glass microfiber GF/C filter (Whatman) by vacuum filtration using a Whatman membrane filter holder apparatus (Millipore Corporation), washed with 20 ml of ice-cold 5% (w/v) TCA followed by 5 ml of ethanol, and the filters dried and counted for ^{3}H in 10 ml of scintillant (Emulsifier-safe; Packard, Downers Grove, IL).

[29] Assay and Expression of Mitogen-Activated Protein Kinase, MAP Kinase Kinase, and Raf

By DARIO R. ALESSI, PHILIP COHEN, ALAN ASHWORTH, SALLY COWLEY, SALLY J. LEEVERS, and CHRISTOPHER J. MARSHALL

Introduction

Raf protein kinase, mitogen-activated protein (MAP) kinase kinase (MAPKK), and MAP kinase (MAPK) lie directly downstream of p21^{ras} in a signal transduction pathway that is activated by growth factors and plays a crucial role in cell proliferation and differentiation. The role of p21^{ras} is to recruit Raf to the plasma membrane,[1,2] where it is activated by an as yet unidentified mechanism. Raf activates MAPKK by phosphorylating it at Ser-217 and Ser-221[3] and MAPKK then activates MAPK by phosphorylating Thr-183 and Tyr-185.[4] In this chapter we describe procedures for the assay, expression, and purification of these three kinases.

[1] S. J. Leevers, H. F. Paterson, and C. J. Marshall, *Nature (London)* **369**, 411 (1994).
[2] D. Stokoe, S. G. Macdonald, K. Cadwallader, M. Symons, and J. F. Hancock, *Science* **264**, 1463 (1994).
[3] D. R. Alessi, Y. Saito, D. G. Campbell, P. Cohen, G. Sithanandam, U. Rapp, A. Ashworth, C. J. Marshall, and S. Cowley, *EMBO J.* **13**, 1610 (1994).
[4] D. M. Payne, A. J. Rossomando, P. Martino, A. K. Erikson, J. H. Her, J. Shabanowitz, D. F. Hunt, M. J. Weber, and T. W. Sturgill, *EMBO J.* **10**, 885 (1991).

Buffers

Buffer A: 50 m*M* Tris-HCl (pH 7.5, 20°) 0.1% (by volume) 2-mercaptoethanol, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.1 m*M* tosyl phenyl chloromethyl ketone, and 1 m*M* benzamidine

Buffer B: 20 m*M* Tris/acetate (pH 7.5, 20°), 0.27 *M* sucrose, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* sodium orthovanadate, 10 m*M* sodium *β*-glycerophosphate, 50 m*M* sodium fluoride, 5 m*M* sodium pyrophosphate, 1% (by mass) Triton X-100, 0.1% (by volume) 2-mercaptoethanol, 1 m*M* benzamidine, 0.2 m*M* PMSF, and leupeptin (5 μg/ml)

Buffer C: 50 m*M* Tris-HCl (pH 7.5, 20°), 0.03% (by mass) Brij 35, 0.1 m*M* EGTA, and 0.1% (by volume) 2-mercaptoethanol

Buffer D: 50 m*M* Tris-HCl (pH 7.5), 0.03% (by mass) Brij 35, 0.1 m*M* EGTA, 0.1% (by volume) 2-mercaptoethanol, 0.66 μM okadaic acid (to inhibit protein phosphatases 1 and 2A), 0.27 m*M* sodium orthovanadate (to inhibit protein tyrosine phosphatases), 13.3 m*M* magnesium acetate, and 0.33 m*M* ATP.

Buffers A to D are stored in the absence of 2-mercaptoethanol and proteinase inhibitors, which are added from concentrated stocks immediately before use. Benzamidine and leupeptin are stored at −20° in water and phenylmethylsulfonyl fluoride is dissolved in ethanol just before use.

Measurement of Protein Concentration. Protein concentration is determined by the method of Bradford,[5] using bovine serum albumin as the standard.

Assay of MAPK

Principle and Definition of Unit. MAPK is assayed by its ability to phosphorylate myelin basic protein (MBP). One unit of activity is that amount of enzyme that incorporates 1 nmol of phosphate into MBP in 1 min. The MAPK concentration in units per milliliter (U/ml) is calculated using the formula 25 *CD/S*, where *C* is the ^{32}P radioactivity incorporated into MBP (cpm), *D* is the fold dilution of the MAPK solution before assay, and *S* is the specific radioactivity of the ATP (cpm/nmol).

Reagents. All reagents are kept at 0–4° unless stated otherwise. Myelin basic protein is purchased from GIBCO-BRL (Gaithersburg, MD), dissolved in water to give a 3.3-mg/ml solution, and stored in aliquots at −20°. We recommend that MBP and okadaic acid be purchased from GIBCO-BRL. GIBCO-BRL MBP gives at least five-fold higher activity than the Sigma (St. Louis, MO) product in the MAPK assay, and the potency of okadaic acid as a phosphatase inhibitor from several other commercial sources is poor. Sodium orthovanadate is dissolved in water, adjusted to pH 10, and heated for 10 min at 100°. The pH is readjusted to 10 and this cycle repeated once more before dilution with water to give a final concentration of 0.1 *M*. This procedure depolymerizes the vanadate, converting it into a more potent inhibitor of protein tyrosine phosphatases.[6] The 20-residue peptide TTYADFIASGRTGRRNAIHD (PKI), a specific inhibitor of cyclic AMP-dependent protein kinase, can either be synthesized or purchased from Sigma.

Procedure

Reactions are carried out in 1.5-ml plastic microcentrifuge tubes. MAPK diluted in ice-cold buffer B containing bovine serum albumin (1 mg/ml) (5 μl) is incubated for 3 min at 30° with 35 μl of 36 m*M* Tris-HCl (pH 7.0, 20°), 0.1 m*M* EGTA, and MBP (0.47 mg/ml) and the reactions initiated by the addition of 10 μl of 50 m*M* magnesium acetate–0.5 m*M* [γ-^{32}P] ATP (~200,000 cpm/nmol). After 10 min at 30° the reaction is terminated by pipetting 40 μl of the assay mixture onto a 2 × 2 cm square of phosphocellulose paper (P81; Whatman, Clifton, NJ) that binds MBP but not ATP, and immersing the paper in a beaker containing 0.5% phosphoric acid (5 ml/paper). After washing the papers five times with phosphoric acid to remove ATP (1 min for each wash), followed by one wash in acetone to remove phosphoric acid, the P81 papers are dried with a hair drier and inserted into 1.5-ml plastic microcentrifuge tubes. One milliliter of scintillant is added and the tubes are analyzed for ^{32}P radioactivity. Control incubations are carried out in which MAPK is replaced by dilution buffer, and this value is subtracted from the value obtained in the presence of MAPK. MAPK activities are linear with time up to an activity of 6 U/ml in the assay, and the kinase concentration is therefore kept below this value.

Activity Measurements of MAPK in Tissue Extracts

It is not possible to measure MAPK activity accurately in most cell lysates by the phosphorylation of MBP, because the presence of other kinases that phosphorylate this protein usually interferes with the assays. Only in PC12 (rat adrenal pheochromocytoma) cells, which contain particularly high levels of MAPK, are the p42 and p44 isoforms of MAPK the dominant MBP kinases after growth factor stimulation. In unstimulated PC12 cells p42 MAPK and p44 MAPK are inactive, and activity is therefore

[5] M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).

[6] J. Gordon, this series, Vol. 201, p. 477.

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Buffer C: 50 m*M* Tris-HCl (pH 7.5, 20°), 0.03% (by mass) Brij 35, 0.1 m*M* EGTA, and 0.1% (by volume) 2-mercaptoethanol

Buffer D: 50 m*M* Tris-HCl (pH 7.5), 0.03% (by mass) Brij 35, 0.1 m*M* EGTA, 0.1% (by volume) 2-mercaptoethanol, 0.66 μ*M* okadaic acid (to inhibit protein phosphatases 1 and 2A), 0.27 m*M* sodium orthovanadate (to inhibit protein tyrosine phosphatases), 13.3 m*M* magnesium acetate, and 0.33 m*M* ATP.

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[5] M. M. Bradford, *Anal. Biochem.* 72, 248 (1976).

Alternative Assays for MAPK

The activation of MAPK by growth factors has also been assessed in cell extracts by several other procedures. These are useful but have the limitations and potential dangers described below.

Decreased Electrophoretic Mobility. The phosphorylation of MAPK by MAPKK is accompanied by a decrease in its electrophoretic mobility on sodium dodecyl sulfate (SDS)–polyacrylamide gels and this can be detected in cell extracts by immunoblotting with suitable anti-MAPK antibodies. This assay has been widely used to detect activation, but is only semiquantitative and unsuitable for detecting low levels of activation of MAPK. Reliable separation of the phosphorylated and dephosphorylated forms of MAPK requires 20-cm-long gels containing a final concentration of 10% (w/v) acrylamide–0.166% (w/v) bisacrylamide.[8] There is also the potential danger that the electrophoretic mobility may be decreased by phosphorylation at sites other than those labeled by MAPKK, rendering the assay invalid. This is a particular concern when the activation of MAPK is being studied using a stimulus whose effects on MAPK phosphorylation have never been analyzed in molecular detail (e.g., heat shock). Care is also needed because singly phosphorylated MAPK runs in almost the same position as the diphosphorylated form.

Immunoblotting with Anti-Phosphotyrosine Antibodies. The activation of MAPK is accompanied by the phosphorylation of a tyrosine residue, which can be detected by immunoblotting with a suitable anti-phosphotyrosine antibody. Like the previous method, this assay is semiquantitative and unsuitable for detecting low levels of MAPK activation. A potential danger is that mammalian cells contain other MAPK homologs, which are components of distinct signaling pathways, and are similar in size to p42 MAPK and p44 MAPK.[10,11] These homologs are also activated by a dual threonine/tyrosine phosphorylation mechanism in response to stimuli reported to activate p42 MAPK and p44 MAPK in some cells. A further potential hazard stems from the requirement of threonine phosphorylation, as well as tyrosine phosphorylation, for activity. Tyrosine-phosphorylated MAPK may sometimes be inactive if the threonine residue is dephosphorylated preferentially.

"In Gel" Kinase Assay. In the in-gel assay, which is described elsewhere in this volume,[12] MAPK is denatured by dissolving cell extracts in SDS,

[10] J. M. Kyriakis, P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett, *Nature (London)* **369**, 156 (1994).

[11] J. Rouse, P. Cohen, S. Trigon, M. Morange, A. A. Llanmazares, D. Zamanillo, T. Hunt, and A. Nebreda, *Cell (Cambridge, Mass.)* **78**, 1027 (1994).

[12] C. J. Marshall and S. J. Leevers, this volume [28].

measured by subtracting the MBP kinase activity in lysates from unstimulated cells from that measured in lysates of growth factor-stimulated cells.[7]

In other cell extracts MAPK is measured after immunoprecipitation of the p42 and/or p44 isoforms of MAPK from the lysates with noninhibitory antibodies. We routinely use an antiserum raised against the C-terminal peptide of p42 MAPK (EETARFQPGYRS), which immunoprecipitates p42 MAPK specifically.[8] Cell lysate (5 to 30 μl), prepared as described below, is incubated for 90 min at 4° on a shaking platform with 5 μl of protein A-Sepharose conjugated to 1–2 μl of p42 MAPK antiserum. The suspension is centrifuged for 1 min at 14,000 *g*, the supernatant discarded, and the immunoprecipitate washed twice with 1.0 ml of buffer B containing 0.5 *M* NaCl, twice with 1.0 ml of buffer C, and then assayed for MBP kinase activity as described above. It is important to check that immunoprecipitation is essentially quantitative by demonstrating that no further MBP kinase activity can be immunoprecipitated from the supernatant obtained from the first immunoprecipitation.

MAPK is dephosphorylated and inactivated by protein phosphatases present in cell extracts.[9] The lysis buffer (buffer B) therefore contains fluoride, pyrophosphate, and β-glycerophosphate to inhibit protein serine/threonine phosphatases (principally protein phosphatase 2A) and orthovanadate to inhibit protein tyrosine phosphatases. It also contains EDTA and EGTA to chelate Mg^{2+} and Ca^{2+}, respectively, and so inhibit MAPKK and divalent cation-dependent proteinases and phosphatases. Cells are lysed in ice-cold buffer B, centrifuged for 5 min at 14,000 *g* (4°), and the supernatants decanted. p42 MAPK is then immunoprecipitated and assayed for MBP kinase activity as described above, except that the reaction is carried out on a shaking platform at 30° to keep the immunoprecipitated MAPK/protein A-Sepharose complex in suspension. If the reactions are not shaken, the measured activities are reduced considerably.

Lysates containing 0.5–2 mg of protein per milliliter can be stored at −80° for at least 1 month with no loss of activity if frozen in liquid nitrogen immediately after cell lysis. Frozen lysates are thawed only once. The assay of MAPK in cell extracts or immunoprecipitates also contains okadaic acid (1 μM) and sodium orthovanadate (0.1 m*M*) to inhibit protein phosphatases, and PKI (1 μM) is included to inactivate cyclic AMP-dependent protein kinase, which phosphorylates MBP efficiently.

[7] N. Gomez, N. K. Tonks, C. Morrison, T. Harmar, and P. Cohen, *FEBS Lett.* **271**, 119 (1990).
[8] S. J. Leevers and C. J. Marshall, *EMBO J.* **11**, 569 (1992).
[9] D. R. Alessi, N. Gomez, G. Moorhead, T. Lewis, M. S. Keyse, and P. Cohen, *Curr. Biol.* **5**, 283 (1995).

and electrophoresed on an acrylamide gel polymerized in the presence of MBP. The MAPK is renatured and MBP phosphorylation initiated by incubating the gel with Mg[γ-^{32}P]ATP. After washing to remove ATP, the position of ^{32}P-labeled MBP is located by autoradiography.[13] This assay assumes that no other MBP kinases comigrate with p42 or p44 MAPK and that the extent of renaturation of MAPK is uniform throughout the gel. The method is rather insensitive, because the extent of renaturation is low, and it is also expensive because of the large amounts of MBP and [γ-^{32}P]ATP that are used.

Assay of MAP Kinase Kinase Activity

Principle and Definition of Unit. MAPKK is assayed by its ability to activate MAPK, which is then assayed by the phosphorylation of MBP. The assay is therefore carried out in two stages. In the first step MAPKK is incubated with inactive p42 MAPK and MgATP, and in the second, the extent of activation of p42 MAPK is measured by enzyme assay. One unit of activity is that amount of enzyme that increases the activity of MAPK by 1 U/min.

Reagents. The reagents are the same as those used in the assay of MAPK, except that an inactive glutathione *S*-transferase (GST)–MAPK fusion protein is also required, the purification of which is described below. This fusion protein is activated by MAPKK with high efficiency and cleavage of GST from the fusion protein is unnecessary. Glutathione-Sepharose is purchased from Pharmacia (Piscataway, NJ).

Procedure. Reactions are carried out in 1.5-ml plastic microcentrifuge tubes and MAPKK is diluted in ice cold buffer B containing bovine serum albumin (1 mg/ml). Fifteen microliters of buffer D containing 1.33 μM inactive GST–MAPK is incubated for 3 min at 30° and the reaction is initiated with 5 μl of MAPKK. After 30 min at 30° the activation of GST–MAPK is terminated, and the MAPK assay initiated, by adding 2 μl of the reaction mixture to 48 μl of 25 mM Tris/HCl (pH 7.0, 20°C), 0.1 mM EGTA, MBP (0.33 mg/ml), 10 mM magnesium acetate, and 0.1 mM [γ-^{32}P]ATP (~200,000 cpm/nmol). After incubation for 10 min at 30°, the incorporation of phosphate into MBP is determined as described for the assay of MAPK. A control incubation is carried out in parallel in which GST–MAPK is omitted from the first part of the assay and this reaction blank is subtracted from the value obtained in the presence of GST–MAPK. The activation of GST–MAPK is linear with time up to 6 U of MAPKK

[13] Y. Gotoh, E. Nishida, T. Yamashita, M. Hoshi, M. Kawakami, and H. Sakai, *Eur. J. Biochem.* 193, 661 (1990).

this value.

Measurement of MAPKK in Cell Extracts. MAPKK can be measured in lysates prepared from virtually all growth factor-stimulated cells without the need for immunoprecipitation. Like MAPK, MAPKK is dephosphorylated and inactivated by protein phosphatases (predominantly protein phosphatase 2A and to a lesser extent protein phosphatase 2C)[8] and similar precautions are therefore needed for its assay in cell extracts. Cells are lysed, centrifuged, and stored in the same phosphatase inhibitor-containing solution (buffer B) that is used to assay MAPK in extracts. In addition, okadaic acid (0.5 μM) and vanadate (0.2 mM) are included at both steps of the MAPKK assay, and PKI (1 μM) is included in the second step.

If the activity of MAPKK in cell extracts is low, the assay can be made more sensitive in the following way. After the first stage of the reaction, the activation of GST–MAPK is terminated by adding a 20-μl suspension of buffer B containing 5 μl of glutathione-Sepharose and 20 mM EDTA, pH 7.0. After incubation for 15 min at 4° on a shaking platform, the suspension is centrifuged for 1 min at 14,000 *g*, the supernatant is discarded, and the pellet containing GST–MAPK (attached to glutathione-Sepharose) is washed twice with 1.0 ml of buffer B containing 0.5 M NaCl and twice with 1.0 ml of buffer C. It is then assayed for MBP kinase activity as described above, except that 20 mM glutathione (pH 7.5, 20°) is included in the MBP kinase assay to dissociate p42 MAPK from the glutathione-Sepharose beads. This modified procedure eliminates all interfering MBP kinase activity present in the extracts, making it possible to detect low MAPKK activity in a cell lysate.

Assay of Raf in Cell Extracts

Principle and Definition of Unit. Raf is assayed by its ability to activate MAPKK, which is then assayed by the activation of MAPK. The assay is performed in two stages. In the first step Raf is incubated with inactive MAPKK, inactive MAPK, and MgATP. In the second, the extent of activation of MAPK is measured by enzyme assay. One unit of activity is that amount of enzyme that increases the activity of MAPK by 1 U/min.

Reagents. The reagents are the same as those required for the assay of MAPKK, except that an inactive GST–MAPKK1 fusion protein is also needed, the expression and purification of which is described below. This fusion protein is activated efficiently by Raf[5] and cleavage of GST from the fusion protein is unnecessary. Cells are lysed and prepared for the assay exactly as described for MAPK and MAPKK.

Procedure. Raf cannot be assayed in extracts from cells expressing normal levels of this kinase. This is because activated MAPKK, activated MAPK, and other MBP kinases in the lysates interfere with the assay, and in addition other MAPKK activators (such as MEK kinase and Mos)[14] may also be present. For these reasons Raf can be assayed only after immunoprecipitation from extracts. To assay the isoform $p74^{raf-1}$ we use a polyclonal antibody raised in sheep against the C-terminal peptide (CTLTTSPRLPVF) affinity purified on a peptide–Affi-Gel 15 column.[15] Cell lysate (5-100 μl) is added to 5 μl of protein G-Sepharose conjugated to 0.5–1.0 μg of affinity-purified $p74^{raf-1}$ and incubated for 60 min at 4° on a shaking platform. The suspension is centrifuged for 1 min at 14,000 g, the supernatant discarded, and the immunoprecipitate washed twice with 1.0 ml of buffer B containing 0.5 *M* NaCl, twice with 1.0 ml of buffer C, and then assayed as described below. In some cells overexpressing Raf we have found that it is sometimes advantageous to add 20 m*M* *n*-octylglucoside to both the cell lysis buffer and the buffers used to wash the immunoprecipitates.[1]

Fifteen microliters of buffer D containing 0.27 μM GST–MAPKK1 and 1.33 μM GST–MAPK is added to 5 μl of $p74^{raf-1}$ immunoprecipitate and, after incubation for 30 min at 30° on a shaking platform, a 2-μl aliquot is added to 48 μl of 25 m*M* Tris-HCl (pH 7.0, 20°), 0.1 m*M* EGTA, MBP, (0.33 mg/ml), 10 m*M* magnesium acetate, 0.1 m*M* [γ-^{32}P]ATP (200,000 cpm/nmol). After incubation for 10 min at 30°, the incorporation of phosphate into MBP is determined as described for the assay of MAPK. Two control incubations are carried out in parallel, in which either GST–MAPKK1 or the $p74^{raf-1}$ immunoprecipitate is replaced by buffer C and these reaction blanks are subtracted from the value obtained in the presence of GST–MAPKK1 and $p74^{raf-1}$.

Because the first step of the assay contains both MAPKK and MAPK it is not linear with respect to time, the rate of activation of MAPK increasing progressively as more MAPKK is activated by Raf. Nevertheless, the activation of GST–MAPKK1 after 30 min is directly proportional to the amount of Raf added to the assay up to 2 U/ml, and this concentration of Raf is not exceeded. This assay is extremely sensitive because 2 U/ml corresponds to only a 1.0% conversion of MAPKK1 to its activated form and concentrations of even 0.1 U/ml can be quantified accurately.[3]

This assay can be used without modification to measure other MAPKK activators in cell extracts such as Mos, MEK kinase, and isoforms of Raf following their immunoprecipitation with suitable antibodies. We have also

[14] K. J. Blumer and G. L. Johnson *Trends Biochem. Sci.* **19**, 236 (1994).
[15] A. Hiraga, B. E. Kemp, and P. Cohen, *Eur. J. Biochem.* **163**, 253 (1987).

used this assay to measure $p74^{raf-1}$ activity in lysates from Sf9 (*Spodoptera frugiperda*; fall armyworm ovary) insect cells in which $p74^{raf-1}$ has been activated by coexpression with v-Ras and the protein tyrosine kinase Lck.[5] Owing to the high level of expression and activity of $p74^{raf-1}$ in lysates derived from these cells no immunoprecipitation is required, and the sensitivity of the assay is such that the extract must be assayed at a final dilution of 50,000-fold.

Assay of Total MAP Kinase Kinase Kinase Activity in Cell Extracts

We have modified the assay for Raf to measure the total MAP kinase kinase kinase (MAPKKK) activity in a cell lysate. Cell extracts are diluted in buffer B containing 1 mg of bovine serum albumin per milliliter and an aliquot (5 μl) is incubated with 15 μl of buffer D containing 0.27 μM GST–MAPKK1. After 30 min at 30° the activation of GST–MAPKK1 is stopped by addition of a 20-μl suspension of buffer B containing 5 μl of glutathione-Sepharose and 20 mM EDTA, pH 7.0. After incubation for 15 min at 4° on a shaking platform, the suspension is centrifuged for 1 min at 14,000 g, the supernatant discarded, and the GST–MAPKK1 (attached to glutathione-Sepharose) washed twice with 1.0 ml of buffer B containing 0.5 M NaCl and twice with 1.0 ml of buffer C. MAPKK activity is then assayed as described above, except that 20 mM glutathione (pH 7.5, 20°) is included in the first step of the MAPKK assay to dissociate GST–MAPKK1 from glutathione-Sepharose. Control incubations are carried out in which either MAPKK1 or cell lysate is omitted, and these (extremely low) values are subtracted from those obtained in the presence of MAPKK1 and cell extract. This procedure eliminates interference from the MAPKK, MAPK, and other MBP kinases present in cell lysates, making it possible to measure accurately even a small activation of the added GST–MAPKK1.

Pitfalls in Assay of Raf

Raf is highly specific and, apart from MAPKK, no substrates have been found that are phosphorylated at significant rates.[16] However, until MAPKK was identified as the physiological substrate in 1992, Raf was assayed using a variety of substrates that are now known to be phosphorylated poorly by Raf or not at all.[16] The activities being measured in Raf immunoprecipitates were therefore probably other growth factor-stimulated kinases present as trace contaminants, because it has been known for

[16] T. Force, J. V. Bonventre, G. Heidecker, U. Rapp, J. Avruch, and J. M. Kyriakis, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1270 (1994).

many years that even essentially homogeneous proteins are frequently contaminated with traces of the protein kinases that phosphorylate them. However, even now, Raf is frequently assayed in immunoprecipitates not by the activation of MAPKK, but by the phosphorylation of MAPKK or Raf itself. These assays are hazardous, because MAPKK and Raf are substrates for many protein kinases, such as MAPK,[17] which may be extremely active. For example, the level of phosphorylation *in vivo* of the residues on MAPKK1 phosphorylated by Raf (Ser-217 and Ser-221) is much lower than those phosphorylated by MAPK (Thr-291 and Thr-385).[3,17] We therefore recommend that Raf be measured by the activation, and not by the phosphorylation, of MAPKK. If Raf is measured by the phosphorylation of MAPKK, it is essential to validate the assay by checking that phosphorylation has actually occurred at Ser-217 and Ser-221 and not at other residues, and/or by showing that a MAPKK in which Ser-217 and Ser-221 are mutated to alanine is not phosphorylated under these conditions.

Expression of GST–MAPK in *Escherichia coli*

A full-length murine cDNA encoding the p42 isoform of MAPK[18] was subcloned into the *Bam*HI site of pGEX-2T plasmid[19] to create a fusion protein with GST at the N terminus and p42 MAPK at the C terminus separated by a linker region containing a cleavage site for thrombin. This construct was transformed into *Escherichia coli* strain BL21 DE3 (pLys S).[20]

Large-Scale Purification of Bacterially Expressed GST–p42 MAPK

Bacteria expressing the GST–p42 MAPK fusion protein are grown at 37° in a 25-liter fermenter until the absorbance at 600 nm is 0.6. The temperature of the culture is then reduced to 25° and isopropyl-β-D-thiopyranoside added to 30 μ*M*. The bacteria are grown for a further 20 hr before centrifugation for 10 min at 4000 g. The bacterial pellet is resuspended at 4° in 300 ml of buffer A containing 2 m*M* EDTA, 2 m*M* EGTA, 1% (by mass) Triton X-100, and 0.25 *M* NaCl, using a hand homogenizer, and frozen by immersion in liquid nitrogen in 50-ml Falcon (Oxnard, CA) tubes. After 5 min, the samples are thawed by immersion in cold water and 50-ml portions are sonicated for 4 min on ice, ensuring that the temperature

[17] Y. Saito, N. Gomez, D. G. Campbell, A. Ashworth, C. J. Marshall, and P. Cohen, *FEBS Lett.* **341**, 119 (1994).
[18] D. Stokoe, D. G. Campbell, S. Nakielny, H. Hidaka, S. J. Leevers, C. Marshall, and P. Cohen, *EMBO J.* **11**, 3985 (1992).
[19] D. S. Smith and K. S. Johnson *Gene* **67**, 31 (1988).
[20] F. W. Studier, *J. Mol. Biol.* **219**, 37 (1991).

of the lysate remains below 4°. The suspension is centrifuged for 30 min at 28,000 *g* and the supernatant decanted and mixed at 4° with 80 ml of glutathione-Sepharose equilibrated in buffer A plus 0.03% (by mass) Brij 35. After mixing end over end for 30 min, the suspension is centrifuged for 5 min at 4000 *g*, the supernatant is discarded, and the resin washed repeatedly with 400 ml of buffer A containing 0.03% (by mass) Brij 35 and 0.125 *M* NaCl until the absorbance at 280 nm of the supernatant decreases to <0.05. The GST–MAPK is then eluted from the resin at ambient temperature with three 80-ml portions of buffer A containing 0.03% (by mass) Brij 35 and freshly prepared 20 m*M* glutathione, pH 8.0. The eluate is dialyzed against buffer C, and then against buffer C containing 50% (v/v) glycerol and 0.15 *M* NaCl, and stored in aliquots at −20°. The GST–MAPK is about 80% pure as judged by SDS–polyacrylamide gel electrophoresis and 600 mg is obtained from a 25-liter culture, sufficient for >400,000 assays MAPKK or Raf. The enzyme is stable for at least 1 year.

Expression of GST–MAPKK1-H6 in *Escherichia coli*

A full-length cDNA encoding rabbit MAPKK1[21] was expressed in *E. coli* as a GST fusion protein as follows: The 5′ end of the cDNA was generated by PCR so that a *Bam*HI site was present 16 bp in front of the ATG codon. The full-length cDNA was then ligated into *Bam*HI- and *Eco*RI-cut pGEX3X. This construct produces a fusion protein having the amino acids GIPRSA between the factor X cleavage site encoded by pGEX3X and the initiating methionine of MAPKK1. Six histidine residues were also introduced at the C terminus of the GST–MAPKK1 fusion protein and this GST–MAPKK1-H6 construct was transformed into the bacterial strain BL21/DES(pLysS), digested to completion with *Eco*RI, and then partially digested with *Eag*I, which cuts 330 and 10 bp before the termination codon. After gel purification, the longer fragment was ligated to annealed oligonucleotides encoding the three C-terminal amino acids of MAPKK1, the six histidine residues, and a termination codon followed by an *Eco*RI site.

Large Scale Purification of Bacterially Expressed GST-MAPKK1-H6

Bacteria expressing the GST–MAPKK1-H6 are grown in a 25-liter fermenter, induced, and purified on glutathione-Sepharose as described for MAPK. After dialysis against buffer C, the GST–MAPKK1-H6 is concentrated to 50 ml by ultrafiltration through an Amicon 30 membrane (Amicon,

[21] A. Ashworth, S. Nakielny, P. Cohen, and C. J. Marshall, *Oncogene* 7, 2555 (1992).

Danvers, MA), then aliquots are snap frozen in liquid nitrogen and stored at −80°. The GST–MAPKK1-H6 is stable for at least 1 year and can be freeze-thawed at least five times without any loss of activity. The preparations are about 80% pure as judged by SDS–polyacrylamide gel electrophoresis and are used for the assay of Raf described above. About 200 mg of GST–MAPKK1-H6 is obtained from a 25-liter culture, an amount sufficient for 700,000 Raf assays.

The six histidine residues at the C terminus of GST–MAPKK1-H6 were originally added to permit the affinity purification of the enzyme on nickel nitrilotriacetate agarose, as well as glutathione-Sepharose. This is essential to obtain a nearly homogeneous preparation if induction is carried out at 37°, because most of the polypeptides appear to terminate prematurely and little full-length protein is synthesized.[3] However, it has been found subsequently that this problem does not occur at 25°, the yield of full-length GST–MAPKK1-H6 is increased 100-fold, and the second affinity chromatography is unnecessary.

[30] Assay of MEK Kinases

By CAROL A. LANGE-CARTER and GARY L. JOHNSON

Introduction

Mitogen-activated protein kinases (MAPKs) are rapidly activated in response to stimulation of a variety of diverse receptor types including G protein-coupled serpentine receptors and growth factor receptor tyrosine kinases. MAPKS are positively regulated by phosphorylation on tyrosine and threonine by dual-specificity MAP/ERK kinases (MEKs),[1] of which at least three have been cloned.[2–4] MEK kinase (MEKK) is a serine/threonine protein kinase that can phosphorylate and activate MEK-1 independently of Raf family kinases (Raf-1 and B-Raf), the only other known direct activators of MEK-1.[5] Raf-1 and MEKK phosphorylate similar sites on MEK-1 *in vitro* and these sites are phosphorylated *in vivo* following

[1] R. J. Davis, *J. Biol. Chem.* 268, 14553 (1993).
[2] C. M. Crews, A. Alessandrini, and R. L. Erickson, *Science* 258, 478 (1992).
[3] J. Wu, J. K. Harrison, P. Dent, K. R. Lynch, M. J. Weber, and T. W. Sturgill, *Mol. Cell. Biol.* 13, 4539 (1993).
[4] M. J. Russell, C. A. Lange-Carter, and G. L. Johnson, unpublished data (1994).
[5] C. A. Lange-Carter, C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson, *Science* 260, 315 (1993).

raf Oncogenes in Carcinogenesis

Stephen M. Storm, Ulrich Brennscheidt, Gunamani Sithanandam, and Ulf R. Rapp

ABSTRACT

There are three active *raf* genes in man and at least two in *Xenopus* and *Drosophila*. The mammalian c- and A-*raf* genes have 16 coding exons, which span 40 and 20 kb, respectively. B-*raf* is larger and extends over >46 kb. Human c-*raf*-1 maps to chromosome 3p25 and A-*raf*-1 to Xp21. c-*raf*-1 RNA is present in many tissues, while A-*raf* and B-*raf* expression is restricted. A- and c-*raf* encode cytoplasmic *ser/thr* protein kinases of 68 and 74 kDa, which contain three conserved regions (CR). CR1 and 2 are in the amino terminal half, CR1 comprises the presumed ligand binding site, and CR3 represents the carboxy terminal kinase domain. All three genes can be artificially activated by deletions, provided CR3 is preserved. However, only c-*raf*-1 occurs naturally in truncated versions, such as v-*raf* and v-*mil* in the acutely transforming retroviruses 3611-MSV and MH2. *raf* transformation can also be affected by point mutation, suggesting that this mechanism may activate c-*raf*-1 as an oncogene in carcinogenesis.

Key Words: raf genes, *raf* transformation, *Raf*-1, A-*raf*, B-*raf*, and tumorigenicity studies.

I. INTRODUCTION

raf proto-oncogenes encode cytoplasmic serine/threonine specific kinases, which function in mitogen signal transduction from the plasma membrane to the nucleus.[1,2] There are three known active members in the *raf* family of proto-oncogenes, *Raf*-1 (formerly c-*raf*-1), A-*raf*, and B-*raf*. *Raf*-1 was first identified as the cellular homolog of v-*raf*,[3] the transforming gene of 3611 MSV isolated from retroviral transduction experiments.[4] Subsequent screening of cDNA libraries at reduced stringency resulted in the isolation of A-*raf*.[5,6] The most recently identified member, B-*raf*, was discovered as a transforming gene in NIH 3T3 cell transfection assays of human Ewing sarcoma DNA.[7] Amino acid comparisons of *raf* family genes reveal three conserved regions [CR1, CR2, CR3];[8-10] CR1 is a putative regulatory region surrounding a Cys finger consensus sequence, CR2 is comprised of a serine and threonine rich region, and CR3 represents the kinase domain. The deduced amino acid homologies of these three regions are shown in Table 1. *Raf*-1 has been mapped to chromosome 3p25 in humans,[11] a region frequently altered in small cell lung carcinoma (SCLC),[12,13] familial renal cell carcinoma,[14,15] mixed parotid gland tumors,[16] and ovarian cancer.[17] A-*raf* has been localized to the X chromosome between p21 and q11.[5] Pseudogenes Raf-2 (formerly c-*raf*-2) and A-*raf*-2 have been identified at chromosomal positions 4pter (11) and 7p11.4-7q21, respectively.[5]

The *raf* oncogene family shows a high degree of evolutionary conservation. The nucleic acid and amino acid homologies between human and the mouse, rat, chicken, fruitfly (*Drosophila*), and toad (*Xenopus*) *raf* genes are shown in Table 2. A wide variety of mouse tissues have been examined via Northern analysis for steady-state message levels of the three active *raf* genes, and quite different patterns of expression were found.[18] *Raf*-1 was detected in every tissue or cell line tested, with highest RNA levels found in striated muscle, cerebellum, and fetal brain. A-*raf* was more restricted in its expression and showed greater variation among tissues. Highest levels of A-*raf* were seen in tissues of the urogenital system, including epididymis, seminal vesicle, ovary, kidney, and urinary bladder. B-*raf* expression was confined to fewer tissues than either c- or A-*raf*, with high message levels being observed in cerebrum and fetal brain. In addition to 10 and 13 kb transcripts found wherever B-*raf* is expressed, a B-*raf* probe also detects alternate sized RNAs in testes, placenta, and fetal membranes. It is interesting to note that transcripts from all three genes were found in each fetal tissue examined.

Table 1
Amino Acid Homologies between Conserved Regions of Human A-*raf*, B-*raf*, and *Raf*-1

CR1		CR2		CR3	
A-*raf* vs. B-*raf*:	71.1%	A-*raf* vs. B-*raf*:	61.5%	A-*raf* vs. B-*raf*:	75.7%
A-*raf* vs. *Raf*-1:	68.9%	A-*raf* vs. *Raf*-1:	100%	A-*raf* vs. *Raf*-1:	78.0%
B-*raf* vs. *Raf*-1:	64.4%	B-*raf* vs. *Raf*-1:	61.5%	B-*raf* vs. *Raf*-1:	79.1%

Table 2
Overall Homologies between Human *Raf*-1 And *raf* Genes of Other Species

	Percent homology (%)	
	Nucleotide	Amino acid
Rat *Raf*-1	87.7	99.2
Mouse *Raf*-1	87.6	98.8
Chicken c-*mil*	81.6	96.6
Xenopus raf	75.7	92.6
Drosophila raf	67.9	50.4

Tumorigenicity studies in mice as well as transformation in cell culture have been carried out with retroviral constructs containing truncation and/or mutation-activated *raf* genes.[4,19,20-23] Tumors arose most frequently in the hematopoetic lineages followed by pancreatic epithelium and connective tissues.

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II. ROLE OF *RAF* ONCOGENES IN HUMAN TUMORS

A. Normal and Malignant Hematopoetic Cells

It has been shown that a recombinant retrovirus carrying the viral homologs of *Raf*-1 and c-*myc* can immortalize murine macrophages, B lineage, and erythroid lineage cells from bone marrow or fetal liver and the same retrovirus is capable of causing hematopoetic neoplasms, fibrosarcomas, and dysplasia of the pancreatic epithelium in NFS/n mice.[19,21,24] Expression of *Raf*-1 was studied in normal human bone marrow and in various hematopoetic malignancies. *Raf*-1 levels did not differ in the normal human bone marrow when compared with primary blood samples obtained from patients with various leukemias (including AML, AMMolL, ALL, CML).[25] Neither are steady state levels of *Raf*-1 mRNA altered in the human promyelocytic cell line HL-60 by differentiation, proliferation, or inhibition of protein synthesis. In the same study, other human hemopoetic cell lines revealed *Raf*-1 transcription: U937 monocytic leukemia, KG-1 myeloid leukemia, and Burkitt cell lines. *Raf*-1 is also expressed in normal human peripheral granulocytes, monocytes, and macrophages.[26] A cytogenetic study reported 1 case of a translocation (3;14) (p25;q11) among 8 patients suffering from acute type adult T cell leukemia/lymphoma caused by HTLV-1.[27] The breakpoint at 3p25 raised the question of *Raf*-1 involvement in this case, but molecular data from this patient were not shown. A previous study by the same group found trisomy 3 to be the most common chromosome abnormality in these patients.

The influence of interferon-alpha on the expression of several oncogenes in primary cells of CLL was examined using slot blot hybidization technique. No change in the baseline expression level of c-*raf* was found upon stimulation with interferon-alpha.[28]

B. Carcinomas and Solid Tumors

DNA from a human glioblastoma cell line has been used to transform NIH 3T3 cells. The transforming DNA was found to consist of three parts, containing the coding regions of exons 8 to 17 of the human *Raf*-1, an unidentified 5′ end, as well as a 3′ end derived from human DNA sequence and generating abnormal transcripts and a specific protein of 44 kDa. The DNA rearrangement was not detectable in the original glioblastoma cell line, suggesting that the rearrangement occurred during transfection.[29,30] Analysis of the 5′ region of the transforming sequence revealed sequences derived from the lipocortin II gene coding for the first 16 amino-terminal amino acid residues in the lipocortin-*raf* fusion protein.[31] The same group found a large transforming gene in a NIH 3T3 cell transformation assay using DNA from a human Ewing sarcoma cell line. Sequence analysis of this gene uncovered a region highly homologous to but distinct from *Raf*-1 and A-*raf* and was therefore named B-*raf*. As is the case for the other *raf* family oncogenes, B-*raf* appeared to be activated by amino terminal truncation/fusion that thereby consitutively activates its kinase function.[7] In a similar transforming assay of a human primary stomach cancer, a 5′ truncated *Raf*-1 DNA was shown to be the basis for transforming activity.[32] Closer analysis revealed that the transforming DNA encompasses an unknown DNA segment spanning the first 18 kb, fused to *Raf*-1 sequence from exon 6 to 17, and 1.7 kb of unknown DNA at the 3′ end. Transformation could be achieved by using a subclone that contained just the *Raf*-1 portion of this fusion gene. However, cotransfection with other subclones increased the transformation efficiency. The origin and nature of the 5′ part of this transforming gene remains unclear except that it encodes a very hydrophobic polypeptide.[33]

A NIH 3T3 transfection assay using DNA from a cell line established from a radioresistant human larynx carcinoma led to isolation of a 5′ truncated human *Raf*-1 gene. Restriction analysis indicated that the truncation occurred somewhere near exon 10. Again, the DNA subclones comprising the *Raf*-1 portion of the transforming gene were shown to be sufficient for transformation. The karyotypes of the larynx carcinoma cell line showed the absence of a normal autosome 3. Double minutes were found in one karyotype of the transformed NIH 3T3 cell lines, and it has been suggested that they might represent amplified copies of *Raf*-1.[34] Since no genetic changes in *Raf*-1 were detected in the primary cell lines, it is unclear whether *raf* was involved with the initial transformation of these cells. The fact that the tumorigenicity of this cell line in nude mice was decreased when the cells contained an antisense *Raf*-1 expression vector may reflect a requirement for *Raf*-1 in cell maintenance and or division, if presence of *Raf*-1 antisense transcripts was related to the decrease in tumorigenicity at all. An additional point is that the relative radiation resistance of the original human cell line was reduced in the cells transfected with the antisense construct.[35] Whether *Raf*-1 directly or indirectly affects radiation resistance remains to be determined. If, in fact, this phenotype works through *Raf*-1 activation, it would then follow that up-regulation of upstream activators of *Raf*-1 should also generate the radiation-resistant phenotype. Recent experiments in our laboratory have shown *Raf*-1 to be essential for proliferation of NIH 3T3 cells, and that expression of an antisense construct or a kinase negative mutant impairs *Raf*-1 function and reverts the morphology of *raf* transformed cells (Kolch and Rapp, in preparation). In addition, high level expression of full-length *Raf*-1 and PKC cooperate in transforming NIH 3T3 cells (Kolch and Rapp, unpublished), suggesting that in some tumors overexpression of normal *Raf*-1 may contribute to transformation.

Li Fraumeni syndrome is a cancer family syndrome inherited in a dominant fashion, causing multiple neoplasms. Normal skin fibroblasts of a patient suffering from this syndrome were found to be unusually radioresistant. NIH 3T3 cells transfected with DNA from these (noncancerous) fibroblasts were found to cause tumors in nude mice, and *Raf*-1 was found to be the

transforming DNA. Since a v-*raf* probe rather than the full-length human *Raf*-1 cDNA probe was used, the extent of potential 5′ truncation in this case could not be answered.[36] Unfortunately, neither in the case of the stomach cancer nor in the latter study was the primary tumor material examined for the presence of a rearranged *Raf*-1 gene.

In a study of 33 human tumor specimens by the NIH 3T3 cell transformation assay, Stanton and Cooper found activated (5′ truncated) *raf* as the transforming gene in three of the six tumor DNAs that caused foci (with very low frequencies).[37] The tumor DNAs that gave rise to the *raf* transformed fibroblasts were from a breast carcinoma (BR2-215), a lung carcinoid (CA1- 154), and a renal cell carcinoma (RC1138). In all three cases, the recombination occurred within 2.1 kb either in exon 7 or in intron 7. However, none of the primary tumor DNAs showed a rearrangement of the *Raf*-1 locus, and the efficiency of transformation increased dramatically when DNAs from primary transformants were used, indicating that the recombination event happened during transfection.

Since *Raf*-1 has been mapped to chromosome 3(p25) it has been of particular interest to study the role it plays in tumors that show consistent or high frequency loss of 3p, such as SCLC, RCC, ovarian carcinoma, and mixed parotid gland tumors. To further elucidate the potential role of the *Raf*-1 oncogene in lung cancer, RFLPs were used from the region of *Raf*-1 to screen several primary lung cancer samples as well as cell lines for allelic loss of *Raf*-1. Comparing 11 samples of small cell lung cancer to the corresponding normal tissue, a consistent loss of chromosome 3p alleles was found by using the available RFLP probes (including *Raf*-1, erbA-2, and 2 probes detecting anonymous sequences). All informative cases (5) for *Raf*-1 revealed loss of one allele. In addition, 73 human lung cancer cell lines were examined, and the frequency of heterozygosity compared with the normal heterozygosity level for a particular RFLP. A highly significant loss of one *Raf*-1 allele was detected in all 42 SCLC cell lines. The 31 NSCLC cell lines were heterogeneous with respect to the *Raf*-1 locus. These results demonstrate that one allele of *Raf*-1 is consistently lost in SCLC. The allelic loss is the result of a deletion of chromosome 3p in SCLC extending minimally to 3p25. Whether *Raf*-1 can act as a recessive oncogene in this tumor is under scrutiny. In an earlier study, normal sized *Raf*-1 was shown to be expressed in all cell lines of human lung cancers tested, albeit at different degrees. Approximately 60% showed very high levels of *Raf*-1 message, and these levels correlate well with the amount of *Raf*-1 protein detected by Western blotting.[38] Moreover, the intrinsic serine/threonine specific protein kinase activity of *Raf*-1 was found to be constitutively activated as judged from immune-complex kinase assays. Whether the observed activation is a consequence of mutations in the *Raf*-1 gene or results from the presence of other activated oncogenes that work through *Raf*-1 remains to be determined.

Given the results from *in vitro* mutagenesis work with *RAF*-1 cDNAs, which demonstrated mutational activation of full-length *RAF*-1 cDNA,[2,10] it is now important to use RNAse protection assays[39,40] to examine a variety of human tumors and tumor-derived cell lines for small genetic alterations (or point mutations) that may activate *Raf*-1. The sensitivity of this approach allows us to detect single base mismatches in both mouse and human *Raf*-1 messages (Figure 1), providing a valuable tool for screening large numbers of samples. Encouraging work along these lines was recently reported,[41] where point mutations were shown to oncogenically activate protein kinase C (PKC), another serine/threonine kinase that shares considerable structural homology with *raf*.[42]

III. ROLE OF *RAF* ONCOGENES IN TUMORS OF OTHER SPECIES

A variety of animal model systems have been used to help delineate roles for *raf* oncogenes in tumorigenesis. The fact that *raf* family genes exhibit a high degree of evolutionary conservation, with homologues being found in organisms as diverse as insects, birds, amphibians, and mammals, has enabled the use of genetically well-defined species in the investigation of *raf* function(s). *Drosophila melanogaster*, with its considerable genetic and phenotypic data, has been utilized recently to study *raf* proto-oncogenes.

Hybridizations with a kinase domain probe from v-*raf* resulted in the identification of two *Drosophila* homologs of mammalian *raf*, D*raf*-1, and D*raf*-2.[43] D*raf*-1 was mapped to the X chromosome at position 2F5-6, and D*raf*-2 was localized to chromosome 2 position 43A2-5. Functionally, mutants homozygous for a defective D*raf*-1 die as third instar larvae due to abnormalities in continually proliferating cells.[44] Therefore, while required for proper larval development, embryonic D*raf*-1 does not appear necessary for early viability, which may be accounted for by the presence of maternal transcripts. This is supported by the fact that during embryogenesis message levels are highest during the early stages and drop to low steady-state levels, similar to the observation that *src*-related proto-oncogenes are present in high levels as maternal transcripts.[45] In addition, D*raf*-1 minus oocytes fertilized with wild-type sperm never hatched, indicating a need for maternal D*raf*-1. These eggs exhibit a phenotype similar to those with *l(1)pole hole* mutations, and recent work demonstrates that indeed D*raf*-1 and *1(l)pole hole* are identical and operate downstream of a tyrosine kinase receptor, *torso*.[46] These mutants lack structures posterior to the seventh abdominal segment, do not develop the labrum, and have a reduced head skeleton, indicating that *raf* may function in the development of these structures. A recent report[47] demonstrates that D*raf*-1 is required for correct *torso* function. A *torso* gain-of-function phenotype in which the terminal anlagen differentiate, but abdominal and thoracic structures are missing was shown to be suppressed by lack of D*raf*-1. This observation indicates that D*raf*-1 operates downstream of *torso*, consistent with the previous D*raf*-1 studies

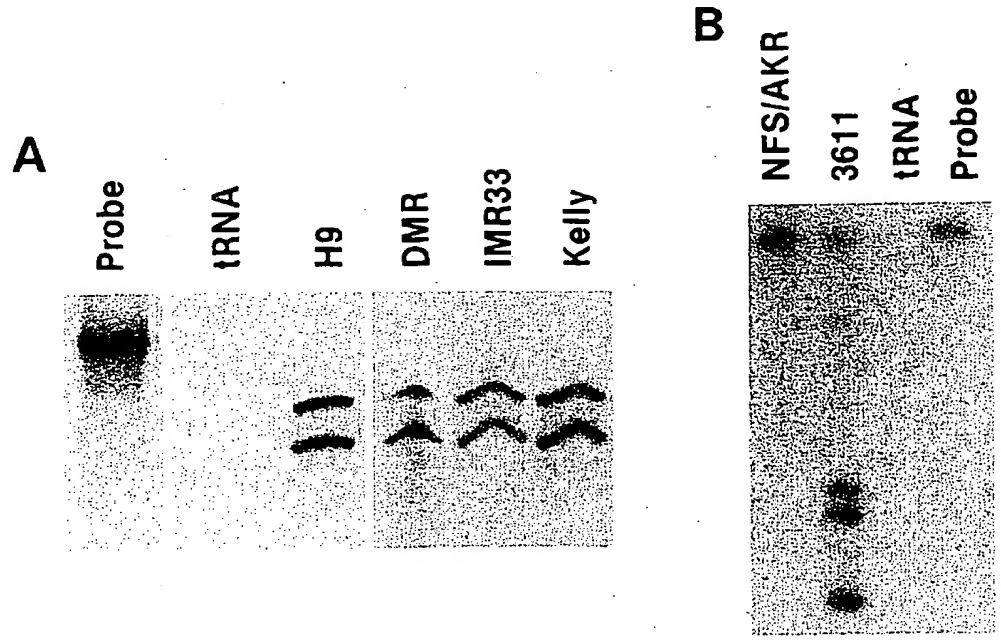

FIGURE 1. RNAse protection assay of human tumor cell lines (A) using a *RAF*-1 antisense probe containing a single point mutation. The twin bands present in each lane demonstrate the ability to detect single base mismatches in *RAF*-1 transcripts using this technique. Part B shows the same method with a wild-type antisense mouse *RAF*-1 probe. Target RNAs are from 3611MSV infected NIH3T3 cells and normal NFS/AKR liver. This wild-type probe also detects single base mismatches as evidenced by the multiple bands present in the 3611 lane, which correspond to single base differences between mouse *RAF*-1 and v-*raf*. There is no probe digestion when hybridized to normal mouse mRNA.

and with mammalian *Raf*-1 functioning downstream of tyrosine kinase receptors.[48,49,74-76] Thus, at least in the *Drosophila* system, *raf* has been shown to be required for signal transduction from a tyrosine kinase class receptor that normally orchestrates development in several distinct pathways. It will be interesting to test whether introduction of activated versions of D*raf*-1 in *Drosophila* will initiate development of tumors in this species.

In the rat, *Raf*-1 is normally expressed as a 73 kDa protein comprised of 648 amino acids with 11 divergent from human *Raf*-1.[50] Rat A-*raf* shows a 59.4% homology with rat *Raf*-1, has a predicted size of 604 amino acids, and a molecular weight of 67.5 kDa.[51] *Raf*-1 is located on rat chromosome 4,[52] and chromosome 4 aberrancies have been observed regularly in chemically induced gliomas and schwannomas[53,54] and in glial cells transformed with MSV.[55] Chromosome 4 also shows considerable banding homology to mouse chromosome 6 (location of murine *Raf*-1), indicating linkage group conservation.[56]

Rat *Raf*-1 was first identified in an oncogenic version through NIH 3T3 cell transfection assays using rat hepatocellular carcinoma DNA, wherein oncogenic activity was acquired during the transfection procedure.[50] In this case, *Raf*-1 appears to have gained its transfection ability through loss of the first 7 exons and recombination with chromosome 13 sequence, resulting in a novel fusion protein. When coupled to the RSV LTR the rearranged cDNA, but apparently not normal *Raf*-1 cDNA, was capable of transforming fibroblasts. While this study did not control for expression of *Raf*-1 protein in cells transfected with normal vs. rearranged cDNA constructs, independent work with human *Raf*-1 cDNA established that up to 10-fold overexpression of normal *Raf*-1 did not overtly transform NIH 3T3 cells, whereas 5′ truncated or specifically mutated versions did.[10,37] The sequence juxtaposed to the 5′ end is likely to be the rat homolog of *tpr* (*t*ranslocated *p*romoter *r*egion),[57] since it shows a 90.1% homology to human *tpr* at the amino acid level.[58] *tpr* has also been shown to recombine with *met* in a similar fashion, resulting in an oncogenically active product.[57,59] The contribution of this upstream sequence on the transforming ability of the truncated *Raf*-1 remains to be determined.

In normal liver *Raf*-1 RNA levels increase approximately fourfold 20 h following a partial hepatectomy and this increase in message coincides with peak DNA synthesis.[60] Diethylnitrosamine-induced rat liver tumors promoted with phenobarbitol exhibited elevated levels of *Raf*-1 message in approximately 85% of either neoplastic nodules or hepatocellular carcinomas.[61] However, in these tumors *raf* expression did not cor-

relate with histone H4 expression, nor did *Raf*-1 expression relate to the malignancy of the tumors tested. This indicates that in these tumors *raf* overexpression is not simply a product of or required for increased DNA synthesis or cell proliferation and suggests that *raf* may be playing another role in these tumors. Induction of *raf* expression appears to be specific for *Raf*-1 as levels of A-*raf* were not altered.

The coding sequence and chromosomal location have been determined in the mouse for both A-*raf* and *Raf*-1. *Raf*-1 maps to chromosome 6 (which shares many similarities with rat chromosome 4, see above)[62] and its 3.2 kb message encodes a 72 kDa serine/threonine protein kinase. A-*raf* is located on the X chromosome,[63] as in rat and human, and a 68 kDa protein is generated from its 2.6 kb message.

The oncogenically active v-*raf*, derived from mouse *Raf*-1, is capable of transforming a variety of cell lines *in vitro* and inducing a defined spectrum of tumors *in vivo*. Newborn mice inoculated intraperitoneally with the v-*raf* expressing 3611 MSV develop fibrosarcomes, erythroblastosis, and occasionally erythroleukemia.[19,64] Of inoculated animals, 100% develop one or more of these malignancies with a latency period of 4 to 8 weeks. The first detectable lesions in these mice are clusters of malignant fibroblasts on the diaphragm, which metastasize throughout the peritoneal cavity and invade the spleen. In addition to the above-mentioned tumors, histological examination of these animals revealed that 3611-MSV inoculation also results in foci of pancreatic acinar cells, which are seen throughout the parenchyma. These tumors are detectable as soon as 15 d following injection of 3611 MSV. Susceptibility to 3611 MSV induced tumorigenesis is highest for newborns and rapidly decreases as mice become weanlings. It has been demonstrated that this resistance of older mice to 3611-MSV-induced erythroleukemias is controlled by a different genetic locus or loci than is responsible for resistance to *raf*/*myc*-induced lymphomas.[65] Additionally, chickens infected with either the v-*myc* expressing MC29 or MH2 that expresses v-*myc* and the avian homolog of v-*raf*, v-*mil*, develop pancreatic carcinomas.[66]

Truncation-activated A-*raf* and *Raf*-1 have also been tested for their ability to induce murine tumors *in vivo*.[4,67] In these experiments, a 5′ truncated version of either human *Raf*-1, human A-*raf*, or murine A-*raf* was fused in frame to a 3′ deleted v-*raf* in the viral backbone of 3611 MSV. The tumors generated by these viruses were analogous to those produced by v-*raf*, indicating that homologous sequences of either A-*raf* or *Raf*-1 can substitute for v-*raf* in *in vivo* transformation and demonstrates that point mutation differences between v-*raf* and truncated *Raf*-1 are not necessary for oncogenic activity. However, viruses expressing full-length c- or A-*raf* are non-tumorigenic, showing that overexpression of the wild-type protein is insufficient for transformation. On the other hand, site-specific alterations have been shown to activate *Raf*-1 as a transforming gene.[38] These experiments were performed by producing linker insertion mutations in CR2 of *Raf*-1 with the resulting construct capable of transforming fibroblasts *in vitro*. This information is important in assessing whether or not *raf* may be oncogenically active in a particular tumor as the presence of only normal-sized transcript and protein does not necessarily indicate a lack of transforming *raf*, since these mutant products are of a wild-type size. As mentioned above for human tumors, the status of *raf* in murine tumors is currently being examined through RNAse protection experiments.

IV. DISCUSSION

Structure-function analysis of human *Raf*-1 and A-*raf* in this laboratory[2,6,8,10] and by others,[29,31,33,37] as well as the observed analogy of *raf* kinase to protein kinase C[42,68] has led us to propose the following working hypothesis for activity regulation of *raf* protein. Wild-type *raf* consists of an amino terminal regulatory domain and a carboxy terminal catalytic domain. Upon ligand binding or specific phosphorylation the catalytic region becomes accessible to substrates via a conformational change in the protein. Oncogenic effects of *raf* seem to result from constitutive activation of kinase activity. This may be achieved either by amino terminal truncation (removal of the regulatory domain) with or without fusion to another sequence, or by specific mutations that alter the conformation of the protein or by steric distortion of the *raf* protein due to N-terminal fusion of intact *raf* with another protein.

Evidence for transient, phosphorylation-mediated activation of *Raf*-1 by a variety of mitogens comes from experiments that showed *Raf*-1 kinase stimulation by the PKC activator TPA, growth factors for transmembrane tyrosine kinase receptors, transformation of cells by tyrosine kinase oncogenes or by *raf* oncogenes.[48] At least two independent mechanisms exist for *raf* recruitment: one involving PKC and another that may depend on binding of *Raf*-1 protein to activated receptor tyrosine kinases[48,49] or direct tyrosine phosphorylation in the case of receptors coupling to intracellular non-receptor tyrosine kinases.[74-76] Whether this binding interaction is in itself sufficient for *Raf*-1 activation by alteration of *Raf*-1 protein conformation or depends on receptor-mediated activation of other events such as generation of activating ligands from stimulated lipid turnover, for example, remains to be established. These results are in agreement with D*raf*-1 functioning downstream of the tyrosine kinase receptor *torso* in *Drosophila*. Moreover, upon activation by either PDGF or TPA, *Raf*-1 migrates from a diffuse cytoplasmic localization to the perinuclear space and the nucleus.[2] *raf* substrates have not been identified to date; however, it was demonstrated that *Raf*-1 can activate PEA-3/AP-1 dependent transcription.[69,70] Whether this is a direct effect of *raf* or is mediated by other components is unknown. The above data strongly suggest that *raf* normally functions in transducing mitogenic signals from the cell surface to the nucleus.

There are limited data concerning the involvement of *raf* family genes in primary tumors. Wherever an activated *raf* gene has been isolated from primary tumor DNA it appears to have been altered as a result of transfection, because no altered

raf gene has been found in the original tumor DNA. Moreover, transfection of DNA isolated from normal tissue has yielded truncation-activated *raf*. Some authors argue that *raf* may be present in an activated form in a subpopulation of cells in these tumors, although this possibility has never been thoroughly examined.

The fact that rearrangements or deletions consistently occur within the same limited region of *Raf*-1 (around intron 7) in both human and rat indicates that this region may be particularly susceptible to breakage with subsequent activation of *raf*. Further data are needed on the level of primary tumors in order to evaluate the presence of various forms of activated *raf* genes, i.e., truncations and point mutations *in vivo*.

In some human lung cancer cell lines there is increased *Raf*-1 kinase activity (see above). Whether the increased kinase activity in these tumor cell lines is due to upstream effects in the signaling pathway, loss of negative regulators of *raf* activity, or due to structural changes of the *Raf*-1 protein caused by mutations on the DNA level is under investigation. The heterogeneity for the loss of a *Raf*-1 allele in NSCLC is consistent with data obtained by others that demonstrated an infrequent loss of 3p sequences in NSCLC.[71] Further work has to address the question whether the loss of *Raf*-1 alleles in a subgroup of NSCLC has clinical implications.

In addition to human lung carcinomas, 3p deletions have been observed in human renal cell carcinomas[14,15] and tumor tissue from patients suffering from von Hippel Lindau disease.[72] RFLP analysis of the *Raf*-1 locus showed allelic loss in all informative cases of von Hippel Lindau disease and sporadic renal cell carcinoma examined.[77] Whether the retained *Raf*-1 allele is oncogenically activated in these cases has not yet been determined.

raf genes may also have clinically important roles apart from direct involvement in tumorigenesis. Transformation of rat liver epithelial cells with v-*raf* or v-H-*ras* was able to confer multidrug resistance and increase the expression of MDR-1 (*P*-glycoprotein) and glutathione-*S*-transferase,[73] both of which are believed to be involved in the multidrug-resistant phenotype. This suggests that *raf* may play a role in generation of resistance and could be exploited clinically.

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The Role of Raf Kinases in Development and Growth of Tumors

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Introduction

Cancer is a disease that is caused predominantly by genetic alterations. A critical group of genes involved in malignant transformations includes the so-called oncogenes. There are four categories of protooncogenes from which oncogenic products can emerge: (1) peptide factors that act as ligands for cell surface receptors, (2) the receptors themselves, (3) intracellular signal transducers, and (4) transcription factors. Mutations of oncogenes leading to cancer frequently cause constitutive activity of the gene products. This results in an unregulated and enhanced transduction of mitogenic signals from the cell membrane to the nucleus.

Raf proteins belong to the third category of protooncogene products. They are serine/threonine kinases that are now known to play a central role in mediating the mitogenic response of cells to numerous growth factors and cytokines. Once a receptor is activated by its extracellular ligand, the signal is transported via cytoplasmic kinase cascades to the nucleus where transcription of specific genes is induced through phosphorylation and activation of transcription factors. Long-term cellular behavior like suppression of apoptosis, proliferation, and differentiation are regulated by those signaling events. Currently three distinct kinase cascades are known in vertebrates, but others may yet be found (Cano and Mahadevan 1995). The best-understood and the only clearly growth-regulatory cascade is the Ras/Raf/MEK/ERK pathway (Fig. 1). Cytokine receptors as well as receptor-protein tyrosine kinases (RPTKs) link to this pathway via activation of the Ras protein, a protooncogene that is found altered in more than 75% of colon cancers and about 30% of all human cancers (Ando et al. 1991; Boland 1993). Ras activation is achieved by translocation to the plasma membrane of the Grb-2/Sos complex that binds to an autophosphorylation site in the RPTK itself, or to a substrate or docking protein phosphorylated by a nonreceptor protein tyrosine kinase. Juxtaposition of Sos and Ras at the plasma membrane results

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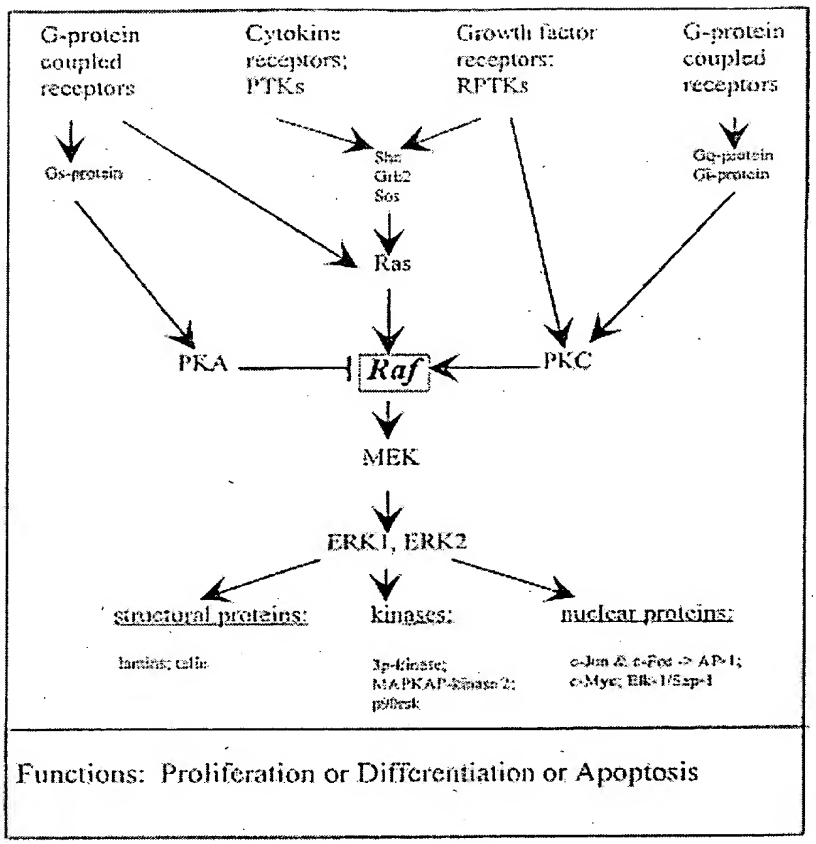

Fig. 1. Simplified model of Raf-dependent signal transduction. Raf is activated upon stimulation of a variety of receptors and, together with MEK and ERK, forms a cytoplasmic kinase cascade. ERKs act on a panel of targets that finally regulate important cellular functions. *Arrows* indicate direct or indirect activation, *blocked lines*, inactivation

in exchange of GDP for GTP on Ras. Only the GTP-bound form of Ras is able to bind to an N-terminal sequence of Raf, termed the Ras-binding-domain (RBD), thus recruiting Raf to the membrane. There, an as yet uncharacterized event activates Raf, which subsequently phosphorylates and activates MEK, which in turn phosphorylates and activates the MAP-kinases ERK1/ERK2. In contrast to Raf and MEK, both of which are able to recognize only one substrate, MAP-kinases can activate a panel of target proteins as indicated in Fig. 1 (reviewed in Daum et al. 1994).

Raf and Cancer

There are several lines of evidence that point to the role of Raf kinases in malignant transformation. Raf first came into view as part of an acute transforming murine virus (Rapp et al. 1983). Furthermore, transforming versions of *raf* genes have been detected in fibroblasts following transfection with DNA from various tumor cells including primary human stomach cancer cells (Shimizu et al. 1985), a human glioblastoma cell line (Fukui et al. 1985), cells derived from renal and breast carcinoma and a lung carcinoid (Stanton and Cooper 1987) as well as chemically induced rat hepatocarcinoma cells (Ishikawa et al. 1985, 1986, 1987). The oncogenic mutations detected were 5′ deletions of *c-raf-1* resulting in N-terminally truncated or fused Raf proteins. However, the mutations could not be detected in the primary tumors and it appears that the oncogenic Raf versions were generated by DNA breakage during transfection (Ishikawa et al. 1986, 1987; Stanton and Cooper 1987).

A function of Raf in tumor development was examined on the level of chromosomal aberrations and cellular expression. There are three functional *raf* genes known in vertebrates, called *A-raf*, *B-raf* and *c-raf-1*. In mice *raf* genes are differentially expressed in tissues such that *A-raf* is present in urogenital tissues, *B-raf* is most abundant in cerebrum and testes, while *c-raf-1* is ubiquitously found in all tissues (Storm et al. 1990; Wadewitz et al. 1993). Little is known about the functional consequences of tissue-specific Raf expression and extensive research is in progress to elucidate isozyme-specific Raf effects. In humans as in mice the three functional *raf* genes are located on different chromosomes. Human *A-raf* is located on chromosome X region p11.2, *B-raf* on 7q34, and *c-raf-1* on 3p25. The chromosomal region Xp11.2 is known to be altered in a variety of human diseases, e.g., Norrie's disease, Wiskott-Aldrich-syndrome, and Cone dystrophy (Bleeker-Wagemakers et al. 1985; Kwan et al. 1988). However, no functional correlations between these diseases and alterations of the *A-raf* gene locus have been described so far. Alterations in 3p25 were observed in familiar renal carcinomas, certain salivary gland tumors, and ovarian carcinomas (Rapp et al. 1988). In small cell lung cancer (SCLC), loss of heterozygosity was frequently found in chromosome 3p regions involving the *c-raf-1* gene in 80% of analyzed tumor tissues. Along with this phenomenon Raf-1 appears to be constitutively activated (Sithanandam et al. 1989; Graziano et al. 1991).

In order to study *c-raf-1* as a potential target in lung carcinogenesis we have designed a mouse tumor model for rapid induction of lung tumors. Tumors were induced by in utero exposure of F1 mice from NFS x AKR matings to 1-ethyl-1-nitrosourea (ENU) on day 16 of gestation. This strain combination was expected to be particularly susceptible to induction of lung tumors and lymphomas based on earlier work by Diwan and Meier (1974). Tumor promotion was achieved by treating weanling mice with weekly intraperitoneal injections of the antioxidant butylated hydroxytoluene BHT which has been shown to cause lung lesions and hyperplasia in mice (Witschi and Saheb 1974).

In this system nearly 100% of the offspring developed lung adenocarcinomas and 70% additionally developed T-cell lymphomas. When tumors were examined for altered expression or structure of tumor-associated genes it was found that one allele of *c-raf-1* was consistently mutated in all tumors, along with a conspicuous lack of mutations of the Raf-activator Ras. Furthermore, no mutations in the tumor-suppressor gene p53, which is known to be altered in many types of human cancers, could be detected (Müller and Naumann, unpublished data). The prevalent mutation in the *raf* gene was an exchange of serine to phenylalanine in position 533 of the kinase subdomain VIII. Additional mutations also clustered in that region, whereas no other mutations could be detected throughout the rest of the Raf molecule. The consistently mutated region apparently forms the surface of the substrate pocket (Fig. 2). This was suggested by computer modeling based on the available coordinates of protein kinase A. Although the mutated allele of *c-raf-1* was not constitutively active, an increased activity of Raf after stimulation by coexpression with Ras

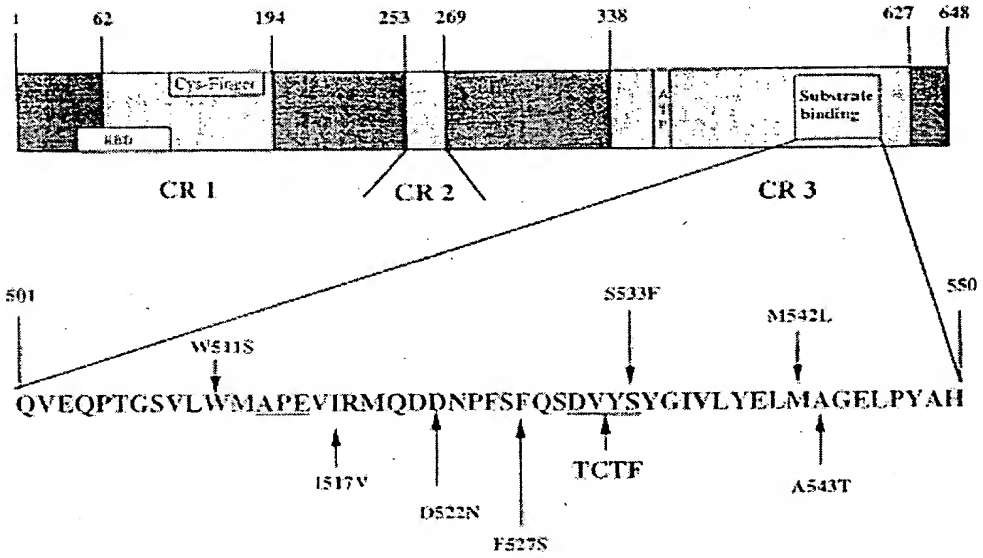

Fig. 2. Clustering of mutations in the murine *c-raf-1* gene after 1-ethyl-1-nitrosourea/butylated hydroxytoluene (ENU/BHT) treatment. Raf family proteins contain three conserved regions named *CR1*, *CR2*, and *CR3*. The N-terminal part of the Raf kinase contains regulatory elements, e.g., the Ras binding domain and a zinc finger motif in CR1, and regulatory phosphorylation sites in CR2 (for details see Daum et al. 1994; Avruch et al. 1994). The carboxy terminal half of the molecule comprises CR3, the catalytic kinase domain. All of the identified mutations cluster in a small area around the APE-site (conserved sequence located in subdomain VIII), apparently at the surface of the substrate pocket. Amino acid sequence is that of wt-murine *c-raf-1* with *arrows* indicating substitutions

and the nonreceptor tyrosine kinase Lck in insect cells could be demonstrated for the most common mutations (Storm et al., in preparation).

In addition to mutational activation of Raf, its level of expression may be a determinant of cellular transformation as suggested by cooperative transformation experiments with wild-type *ras* and *c-raf-1* in NIH3T3 fibroblasts (Cuadrado et al. 1993). Cooperation was only seen when overexpressing Raf together with oncogenic, i.e., constitutively active, Ras expressed at low levels, or wt-Ras expressed at high levels. This indicates that Raf is limiting for Ras-mediated transformation under conditions where only few activated Ras molecules are present at the inner face of the plasma membrane.

In our mouse model Northern and Western blot analysis revealed elevated levels of *c-raf-1* mRNA as well as Raf protein in all tumors compared to normal tissue. Additionally, one member of the *myc* gene family (either *c-*, *N-*, or *L-myc*) was overexpressed in each case. Also, of the *ras* genes at least one member (*Ki-*, *Ha-*, or *N-ras*), and often more than one, was found to be expressed at elevated levels (Storm and Rapp 1993). A synergism between Raf- and Myc-dependent pathways in tumor development has already been described (Rapp et al. 1986). Examination of the role of *raf* genes in human diseases in the future should include the search for point mutations. If clustering of such mutations were observed, similar to the finding in our mouse model, it might be possible to develop inhibitors that can distinguish between normal and oncogenic Raf kinase.

Inhibitors of Raf Kinases

Specific inhibitors are valuable tools for biochemical characterizations of enzymes. There are many gaps in the understanding of the mechanisms of Raf kinase activation and of how activation is regulated. The use of specific Raf inhibitors may elucidate still-unknown regulatory events, and the detection of putative Raf-specific inhibitors might be an important step in the development of anticancer drugs.

The U.S. National Cancer Institute (NCI) natural product database offers growth inhibitory data for approximately 21 000 extracts predominantly derived from plants and fungi that have been tested for anticancer activity in the NCI panel of human tumor cell lines. We tested eight of those extracts (natural products, NPs) for their ability to inhibit the growth of normal and Ras- or Raf-transformed cell lines. The eight extracts were chosen because they had shown growth inhibitory patterns strongly correlating with those that had been obtained in preliminary experiments using antisense *Ki-ras* oligonucleotides. This approach could principally detect inhibitors of either Ras or downstream members of a Ras-dependent signaling cascade. For the growth inhibitory studies we utilized normal NIH3T3 mouse fibroblasts, 3T3 cells transformed with *c-Ha-ras* under the transcriptional control of an SV40 promoter, and 3T3 cells transformed by a mutant *c-raf-1 gene* (lacking the

amino terminal 90 amino acids of wt Raf-1) under the transcriptional control of a Rous sarcoma virus promoter. The Ha-ras-transformed 3T3 cells were found to be more sensitive to growth inhibition by NPs than either wild-type or mutant *c-raf-1*-transformed cells (Housey et al., submitted). Since Raf-1 functions directly downstream of Ras in mitogen-activated signal transduction, we performed in vitro kinase assays to test the ability of the NPs to inhibit activated Raf-1-mediated phosphorylation and activation of MEK. For these experiments we utilized the Raf/MEK/ERK coupled assay system which was described by Housey et al. (submitted). For comparison we included well-characterized specific as well as nonspecific protein kinase inhibitors: H7, tamoxifen, and staurosporine. At final concentrations of 10 to 1000 μg/ml, seven of eight NPs exhibited substantial inhibition of Raf-mediated phosphorylation of MEK (Table 1), whereas H7, tamoxifen, and staurosporine had no or only weak inhibitory effects at comparable concentrations.

Future experiments will attempt to characterize precisely the nature of the inhibitory extracts and to learn about the mechanisms involved. First results point to an interference with Ras/Raf binding in the case of two of the NPs (Housey et al., submitted).

Raf-Deficient Mice

The generation of Raf-deficient mice may provide a means of studying the role of Raf kinases in development and cancer. To prevent the expression of a functional protein, an exon near the 5′ end of the target gene is disrupted by insertion of a marker gene. In case of *c-raf-1* as well as *B-raf* exon 2 was chosen to be interrupted by a neomycine gene (L. Wajnowski and U.R. Rapp, unpublished data). Using standard techniques we were able to generate either *c-raf-1*

Table 1. Inhibition of Raf kinase activity by natural products (Housey et al., submitted)

NP	Organism	Inhibition of Raf-mediated MEK phosphorylation	Concentration (μg/ml)
1	Plant	yes	100
2	Plant	yes	1000
3	Plant	yes	10
4	Plant	yes	10
5	Lichen	yes	1000
6	Plant	yes	10
7	Plant	yes	100
8	Fungus	no	1000

NP, natural product.

or *B-raf*-negative stem cell lines and chimeric mice. Examination of these mice showed that the size of the animals is inversely correlated with the grade of chimerism, indicating a functional role of Raf kinases in embryonic development (L. Wagnowski and U.R. Rapp 1995). Further studies have to await the production of homozygous knockout mice. As to the involvement of Raf kinases in carcinogenesis, it would be interesting to know if mice with Raf-1-deficient lung tissue can be bred and what effect the deficiency might have on lung tumor development after treatment with ENU/BHT as described for our lung tumor model.

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7 Raf protein serine/threonine kinases

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7.1 Introduction

The Raf genes are evolutionarily highly conserved and encode protein serine/threonine kinases with essential function in growth/differentiation-related signal transduction events in organisms ranging from plant to mammals. Genome analysis has revealed the existence of only one functional gene in invertebrates, whereas in vertebrates, three functional (*A-*, *B-* and *c-raf-1*) and several pseudogenes were described. Raf proteins are structurally divided into three conserved regions. CR1 and CR2 contain regulatory elements whereas CR3 represents the catalytic domain. Raf kinases are essential members of intracellular signal transduction pathways. A variety of extracellular signals are transduced by specific receptors to a cytoplasmic kinase cascade consisting of three proteins, Raf, MEK and ERK. The last enzyme of this cascade phosphorylates a diverse array of effectors. This leads to altered gene expression finally resulting in the regulation of cellular processes such as proliferation, differentiation and cell survival. Like many other genes involved in signal transduction, raf genes possesses the hallmarks of a proto-oncogene; deregulated activation of Raf can lead to cellular transformation and tumor induction. This article describes the effects of Raf kinases and their oncogenic forms in the development of cancer, their gene structure, their role and function in signal transduction pathways as well as their function in determination of cell fates.

7.2 Raf: its role in disease

7.2.1 Overview

There are three lines of evidence pointing to a role of Raf in malignant transformation. First, a truncated form is a viral oncogene (*v-raf*). Second, transforming versions have been isolated from cells following transfection with tumor DNA, and third, alterations of RAF gene loci are seen in a variety of human diseases.

The *v-raf* oncogene has been originally identified as a transforming gene of the murine retrovirus MSV3611 [1]. This virus was obtained from a mouse that had developed histocytic lymphoma paralleled by lung adenocarcinoma following infection with an *in vitro* selected variant of the murine leukemia virus (MuLV) at birth, and treatment with the potent carcinogen butylnitrosourea. The avian retrovirus MH2 is another example of a *v-raf*-containing virus. Interestingly, in addition to *v-mil*, the chicken homolog of *v-raf* [2–4], another oncogene, *v-myc*, is also part of this viral genome. MH2 has been isolated from an ovarian tumor in chicken [5] and induces liver and kidney carcinoma in fowl [6, 7].

Further evidence that Raf functions in tumor development was obtained on the level of chromosomal aberrations which can lead to truncated or abnormally regulated genes. In man all three functional raf genes are located on different chromosomes, *A-raf* on chromosome X, region p11.2, *B-raf* on 7q34 and *c-raf-1* on 3p25. Both chromosomal regions containing *A-raf* and *c-raf-1* genes, respectively, are known to be altered in a variety of human diseases, e.g. Norrie disease, Wiskott–Aldrich syndrome, cone dystrophy (all Xp 11.2) [8, 9] and lung and epithelial cancers (3p25) [10, 11]. However, no functional correlations were so far described between these diseases and alterations of raf gene loci.

Besides mutational activation of raf genes, their level of expression may also be a determinant for cellular transformation. This is suggested by transfection experiments in NIH/3T3 fibroblasts, where a cooperative effect between *ras* and wild-type *c-raf-1* was observed [12]. In addition, Storm *et al.*, reported that in mice, *c-raf-1* is overexpressed in lung tumors chemically induced by ethylnitrosourea [13].

Transforming versions of *c-raf-1* have been detected in fibroblasts following transfection with DNA from various tumor cells including a human glioblastoma cell line [14], primary human stomach cancer cells [15], chemically induced rat hepatocarcinoma cells [16, 17], cells derived from renal and breast carcinoma as well as lung carcinoid [18]. The oncogenic mutations detected in four of the described cancer types were 5' deletions of the *c-raf* gene resulting in an N-terminally truncated protein. However, these deletions were absent in the primary tumors suggesting that they occurred after transfection [17–19]. Consistent with the view that truncation of the N terminus as well as overexpression of the Raf-1 protein leads to transformation is the observation that malignant cell lines could be established by cotransfection of NIH/3T3 DNA and long terminal repeat (LTR) sequences of MuLV. It was found that LTR sequences were integrated in exon 5 of the *c-raf-1* gene. That results in high expression of a LTR-U5-Δ-*c-raf-1* hybrid transcript lacking 5'coding sequences corresponding to the conserved region 1 (CR1) [20]. In addition to CR1, deletion of CR2 of the Raf protein may also contribute to cellular transformation (J. Lyons *et al.*, personal communication). In the human breast cancer cell line MCF-7, a deletion in exon 6 of the *c-raf-1* gene effecting the negative regulatory domain was detected (J. Groffen, personal communication).

Recent analysis of a mouse model for rapid induction of lung tumors and T-cell lymphoma revealed the presence of consistent point mutations in one *c-raf-1* allele. All mutations clustered in a region of the catalytic domain with the prevalent mutation being the exchange of Ser533 to phenylalanine. This region apparently forms the surface of the substrate pocket as suggested by computer modeling based on the coordinates of cyclic AMP-dependent protein kinase (see Chapter 2 and [13]). The possibility that point mutations of Raf play a role in tumorigenesis should also be examined in human tumors. All these findings suggest that genetic changes in the *c-raf-1* proto-oncogene leading to its activation or increasing expression levels could contribute to the development of natural and chemically induced tumors. It is noteworthy that none of the other members of the Raf-coupled cytoplasmic kinase cascade (i.e. MAP kinase, protein kinase C, etc.) were found in mutationally activated form in tumors so far.

7.2.2 Raf in retroviruses

7.2.2.1 MSV3611

MSV3611 transforms rodent fibroblast cell lines as well as primary murine fibroblasts [21–23] but not primary bone marrow or fetal liver cells from mice. When primary cells obtained from fetal liver were infected in presence of interleukin 3 (IL-3), the establishment of IL-3-dependent mast cell lines was facilitated. However, additional overexpression of *v-myc* in these cells was required for total IL-3 independence. This demonstrates that *v-raf* has immortalizing functions without abrogating the requirement for IL-3 in myeloid cell lines [24–27].

Infection of newborn NFS/N mice by intraperitoneal injection of MSV3611 results in the development of histiocytic lymphoma, granuloma, fibrosarcoma and erythroleukemia. Although newborn BALB/c and C57BL/6 mice are also susceptible to erythroleukemia upon viral infection, they rapidly acquire resistance to tumor formation when they reach weanling age [28]. Attempts to identify gene loci responsible for this resistance revealed multigenicity and X-chromosomal linkage [29].

7.2.2.2 A-raf MSV

Like *v-raf*, *A-raf* can also act as an oncogene when incorporated into a retrovirus [30]. A constructed virus, 9 IV A-raf MSV, which expresses *A-Raf* as a Gag-Raf-fusion protein was tested for transforming ability in NIH/3T3 fibroblasts. The recombinant virus transformed these cells with an efficiency similar to that of MSV3611 [30]. Intraperitoneal inoculation of newborn NSF/N mice with 9IV A-raf MSV led to a tumor spectrum that overlapped with that of MSV3611 [29] except that 95 % of 9 IV-infected mice additionally developed T-lymphocytic lymphoma (U. Rapp and H. C. Morse, unpublished data).

7.2.2.3 MH2

MH2 causes carcinoma and acute leukemia in fowl. Infection of chicken neuroretina cells with MH2 mutants demonstrated that *v-mil* alone was incapable of causing transformation. Nevertheless, *v-mil* was suggested to play a role in *v-myc*-induced transformation [32–35].

In myeloid cells the transforming activity of *v-mil* is detectable only in the presence of *v-myc* and enables these cells to proliferate in growth factor-deprived medium. It was shown that this was due to the induction of an autocrine loop resulting in the release of growth factors rather than the effect of a downstream bypass [36, 37].

7.2.2.4 The J-type viruses

There are several possibilities by which Raf may interact with Myc in transformation. The functional cooperation of Raf and Myc was examined using viral constructs derived from MSV3611, containing either *v-myc*, *v-raf* or sequences from both oncogenes.

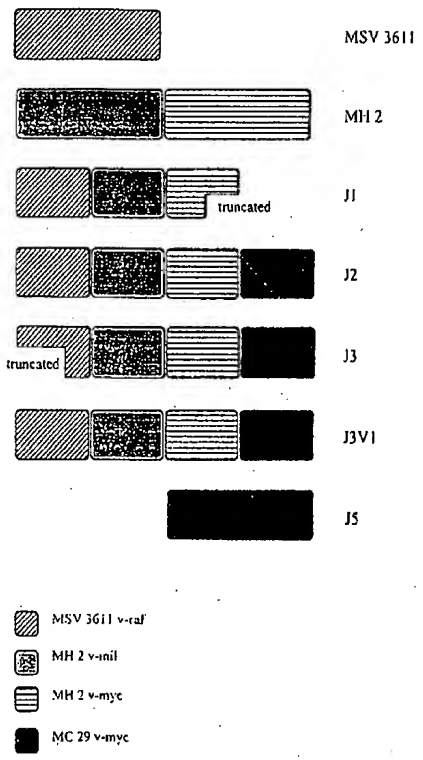

Figure 7.1 Oncogenes in various raf, raf/myc and myc-containing retroviruses. The viral backbone was derived from the Leuk strain of MoMuLV [1, 38].

In the recombinant retrovirus J1, the C-terminal two thirds of MSV3611 *v-raf* are replaced by the corresponding avian *v-mil* sequences (Fig. 7.1). This construct allows to test the effects of 17 out of 19 single amino acid exchanges in which v-Mil differs from murine v-Raf. In J1, the MH2 v-Myc protein is not functionable as it is truncated in its carboxy-terminal half [38]. The J1-virus-induced foci in cell cultures of NIH/3T3 fibroblasts are less pronounced than those induced by MSV3611. Similarly, the average latency of tumors induced in newborn NFS/N mice increases from 6 to 8.5 weeks, when mice were inoculated with J1 as compared with MSV3611. However, the incidence and pathology of all tumors were indistinguishable [24, 38].

The virus J2 (Fig. 7.1) contains *v-myc* in addition to the *raf/mil*-hybrid and this combination endowed it with a dramatically increased ability to transform fibroblasts in culture [38, 39]. Infection of B-lineage and myeloid cells from mouse bone marrow in growth-factor-depleted media resulted in the establishment of cell lines which were neither dependent on nor produced growth factors [24, 26, 39–43].

When newborn NFS/N mice were inoculated with J2 virus, they rapidly showed a variety of neoplasms and all died within 1–3 weeks post-infection. The mice developed a spectrum of tumors that represents not only a summation of malignancies induced by *v-raf* or *v-myc* alone, but also showed additional ones, such as B cell malignancies [44]. As compared with J2, malignancies induced by *v-raf* or *v-myc* alone exhibit a much longer latency [38]. The remarkable strength in transforming activity of J2 is consistent with the model suggested by Cleveland *et al.* in that both oncogenes are part of two different, but synergistic pathways controling cell growth. These were originally termed competence (myc) and progression (raf) pathways [24, 45].

Both, J3 and J5, have a functional *v-myc* gene. Whereas J3 has been derived from J2 by deleting 200 bp from the 5′ end of the raf element resulting in a disruption of the reading frame, J5 does not contain any raf-related sequences (Fig. 7.1) [38, 46]. The histopathology of mice infected with these viruses revealed the development of lymphoblastic lymphoma in 68 % of the animals. Also, a variety of other tumor types such as pancreatic and mammary adenocarcinoma were found [47]. When BALB/c mice were infected with J3 upon treatment with the carcinogen pristane, the induced plasmacytoma differed from those induced by pristane alone, in that they did not carry translocations activating *c-myc* [48, 49]. These data first suggested that deregulated expression of *c-myc* alone is sufficient for the induction of plasmacytoma. Surprisingly, a high proportion of mice infected with J5 upon pristane treatment developed monocyte/macrophage tumors, but rarely, if ever, plasmacytoma. The fact that J3 and J5 carry the same *myc* gene, but cause a different tumor spectrum, prompted the reexamination of the genetic structure of J3 that was reisolated from a plasmacytoma. The analysis revealed two deletion that resulted in the restoration of the original reading frame in the *raf/mil*-hybrid. This J3 variant was named J3V1 (Fig. 7.1) [50]. These findings emphasize the need for genetic changes in addition to deregulation of *c-myc* for induction of plasmacytoma. *V-raf* can provide this missing function.

7.3 Gene structure

The fact that Raf kinases are highly conserved in evolution facilitated the isolation of homologs in a variety of animals and even in the plant *Arabidopsis*. There are three functional genes (*c-raf-1*, *A-raf* and *B-raf*) in vertebrates, whereas only one was described in invertebrates including *Caenorhabditis elegans* and *Drosophila*. In addition to multiple genes, isoforms of Raf kinases can be generated by alternative splicing as reported for the chicken homolog of the *B-raf* and the *c-raf-1* genes.

7.3.1 c-raf genes

Human *c-raf-1* is the best characterized gene of the Raf kinase family. As demonstrated in Fig. 7.2, the human *c-raf-1* gene spans over 70 kbp [51]. The gene consists of 17 exons of which the first is not translated. Intron 1 is at least 25 kbp in size, leaving approximately 45 kbp of genomic DNA containing the translated exons 2 to 17 [52, 53]. The sizes of exons 2 to 16 range from 28 to 233 nucleotides. Exon 17 consists of 141

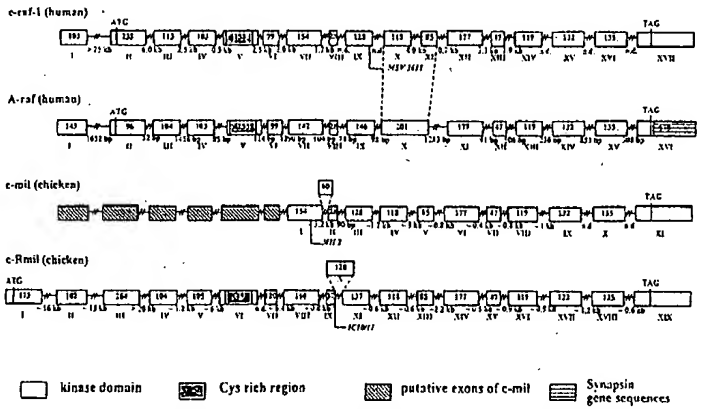

Figure 7.2 Schematic comparison of gene organization of human *c-raf-1*-1, human *A-raf*, chicken *c-mil* and *c-Rmil*. Putative exons of *c-mil* are deduced from cDNA sequence [75. 239] and in comparison with organization of the human *c-raf-1* gene [53]. MSV3611, MH2, IC10/11 show the sites of recombination of the corresponding viruses with viral sequences during retroviral transduction. Arabic numerals indicate the size of exon in nucleotides; roman numerals exon numbers. Translation–initiation and stop codons are indicated.

nucleotides of coding and 905 of non-coding sequences including two polyadenylation signals (AATAAA). Alu-family repeats, typically found in vertebrate DNA, are present throughout the *c-raf-1* gene, except in the immediate vicinity of the last four exons. Characterization of the human *c-raf-1* promoter suggests that it is a housekeeping gene. First, it lacks TATA and CAAT boxes, sequence elements commonly found in inducible eukaryotic genes. Second, it consists of a high percentage of GC (65 %), and third, it contains four GC boxes that are potential binding domains for the transcription factor Sp1 (see Chapter 11). Although the finding that *c-raf-1* is ubiquitously expressed in mouse [54] is consistent with the fact that Sp1 functions in housekeeping genes [55], an argument against this hypothesis is the fact that a TTAA sequence was found 25 bp upstream of one of multiple transcriptional start sites. This element may function similar to a TATAA box as described for the adenovirus EIIa promoter [56].

In addition to the functional *c-raf-1* gene, Bonner *et al.* reported the presence of a pseudogene with 80 % homology, *c-raf-2* [52]. It lacks intron sequences, confirming the hypotheses that pseudogenes are originally generated by reverse transcription of mRNA. *C-raf-2* shows a number of insertions and deletions creating several frame shift and mis-sense mutations.

c-raf-1 homologs have also been cloned from frogs and chickens. A clone obtained from a cDNA library constructed from unfertilized eggs of *Xenopus laevis* consists of an open reading frame of 1.9 kb in size and an 3'untranslated region of 0.7 kb that contains two polyadenylation signals (AATAAA). However, it seems unlikely that either of them is used as both are located far from the poly(A) end. On the contrary, it is suggested that polyadenylation is directed by a rather unusual sequence (ATTAAA) [57, 58]. Interestingly, the 3'untranslated region contains two copies of a sequence element known to decrease RNA stability (ATTTA) [59] as well as two maturation-specific signals (TTTT(A)AT) [60, 61].

The gene structure of *c-mil*, the *c-raf-1* homolog in chicken, is very similar to that of the human gene. It also consists of 17 exons with number 6 to 17 being nearly identical to their human counterparts (Fig. 7.2). Both genes, however, differ in intron sizes which are considerably smaller in the chicken gene [3, 62–64].

7.3.2 A-raf

Incomplete *A-raf* clones were obtained by screening human cDNA libraries using probes derived from *v-raf*. With these clones as probes, a complete clone from a human T-cell library was isolated [30, 65]. This clone was 2452 bp in length with the initiation codon at position 201 preceded by termination codons in all three reading frames. The open reading frame consisted of 1818 nucleotides. The size of the clone corresponded to the 2.6 kb mRNA species seen in Northern blots. The genomic organization of the *A-raf* gene was deduced from two overlapping clones from human placental libraries (Fig. 7.2) [66]. The *A-raf* gene is the smallest of all raf genes described so far. It consists of 16 exons spanning over 10.8 kb genomic DNA. The intron/exon structure of *A-raf* is very similar to that of the human *c-raf-1* gene except that exon 10 and 11 of *c-raf-1* are combined in *A-raf*. All intron/exon borders follow the typical splice junction sequence, except one where a GG is found instead of the consensus AG in the splice acceptor site of exon 13 [66]. The *A-raf* gene contains two Alu-type repeats, both showing highest homology to members of the Alu-S subfamily [67].

The *A-raf* promoter region is localized between nucleotides –59 and +93. In contrast to human *c-raf-1*, the promoter of *A-raf* has a low G/C content [51, 66]. Like *c-raf-1*, the *A-raf* promoter lacks TATAA and CAAT boxes, but interestingly, a motif similar to the E-box is located at the transcriptional start site. This element is known to interact with the immunologically related transcription factor USF and TFII-I [68]. However, it is unknown whether these elements are required for the activation of *A-raf*. In additional to the E-box, multiple steroid hormone responsive elements (glucocorticoid responsive element, GRE/progesteron responsive element, PRE) are present. The motifs GRE1 (position –18) and GRE2 (position –34) are highly conserved between human and mouse, suggesting evolutionary importance of these sequences. Recent experiments have shown that the *A-raf* promoter can be induced by glucocorticoids and dexamethasone in HeLa cells. The glucocorticoid receptor interacts with the GRE/PRE motifs with different affinities: (GRE2>GRE3>GRE1). GRE1 seems to function as a dominant site for hormone induction whereas GRE2 and GRE3 appear to exert an additive effect on GRE1 in presence of hormone. GRE1 and GRE2 are suggested to be required for basal activity of the *A-raf* promoter (J. E. Lee, T. W. Beck, L. Woynowski, U. Rapp, unpublished result). These observations may explain the fact that *A-raf* transcripts are mainly detectable in steroid hormone-responsive tissues [69]. In addition to the functional gene, a pseudogene was found in man with high homology to *A-raf*. It contains several deletions creating termination codons in all three reading frames [66].

7.3.3 *B-raf*

The human *B-raf* gene was first described in a sarcoma where it had been activated by chromosomal rearrangement [70]. Lateron it was cloned by screening human testis cDNA libraries [71, 72]. The *B-raf* gene harbors an open reading frame of 2.3 kb coding for a protein of 766 amino acids. The site of polyadenylation is preceded by two polyadenylation sequences. The human *B-raf* gene contains a 120 bp alternatively spliced exon which has also been described in the avian homolog *C-Rmil*. Additionally, a sequence of 36 nucleotides (exon 8b) suggested to be used as an alternative exon, is located between exons 8 and 9 (J. V. Barnier, personal communication). A high degree of homology is found in the 5' region of chicken *c-Rmil/B-raf* and human *B-raf*. This region is unique for *B-raf* genes [73].

In addition to the *B-raf* gene, a second locus containing homologous sequences is found in the human genome. Sequence analysis of this pseudogene shows alterations including the introduction of stop codons and reading frame shifts, typical for a processed pseudogene [73, 74].

Like human *c-raf-1*, the chicken *B-raf* homolog *c-Rmil* is extremly large in size and spans over more than 100 kbp (Fig. 7.2). The coding region of *c-Rmil* is divided into 19 exons including the 120 bp alternatively spliced exon (exon 10). Exon sizes range from 37 to 264 bp whereas the length of introns highly vary. In the region of the kinase domain the average length of introns is relatively small (0.6–2.2 kb) in contrast to the 5' located introns which are much longer (up to 20 kb). Analysis of the promoter region shows that the chicken *B-raf* gene, like human *c-raf-1*, lacks TATAA and CAAT boxes [75] and has multiple transcriptional initiation sites between positions –13 and –39 upstream of the ATG codon of *c-Rmil* [76]. The major structural differences that distinguish *c-Rmil* from other *mil/raf* genes are found in the 5' region. It contains one additional exon, encoding the first 46 amino acids, whereas the coding region of *c-raf-1* starts in its second exon [53, 71, 72].

7.3.4 *Raf* genes in invertebrates

The *c-raf-1* homolog in *Drosophila melanogaster*, *D-raf*, was isolated by screening genomic and cDNA libraries with a DNA fragment containing parts of the coding region of human *c-raf-1* [77]. Southern blot analysis indicated that only one raf gene is present in the *Drosophila* genome; however, several distantly related genes were described [78]. In contrast to vertebrate raf genes, *D-raf* is very small and contains only three short introns of 64, 68 and 65 bp, respectively. The nucleotide sequences at the splice junctions all agree well with the consensus motif. The promoter region of *D-raf* contains a TATAA sequence, and a putative transcription start site is located 25 bp downstream of the TATAA box. The length of the transcription unit in the longest open reading frame is 2.6 kb. This corresponds well to the actual size of the mRNA of 2.9 kb which may contain a poly(A) stretch of nearly 300 bases. In the isolated cDNA clone, a stretch of three unusual putative polyadenylation signals was found 55 bp upstream of the poly(A) tail.

Only one raf gene, *Ce-raf* was found in the nematode C. elegans and compared with mammalian raf genes, exhibits the highest homology to *c-raf-1*. The *C-raf* gene is devided into 12 exons, and exon/intron boundaries follow the conserved motifs [79].

7.3.5 Raf genes in plants

Recently, a gene coding for a Raf kinase, CTR1, was described in *Arabidopsis*. CTR1 is involved in the ethylene signal transduction pathway. The gene spans over 6.5 kb of genomic DNA and contains 14 introns. The longest ORF is 2466 nucleotides in length and encodes a polypeptide of 821 amino acids with a molecular mass of 90 kDa [80].

7.4 Chromosome mapping of Raf family proto-oncogenes

7.4.1 *C-raf-1*

In man, *c-raf-1* has been mapped to chromosome 3p25 [81]. This site is often altered in several neoplasias [82–84], including sporadic renal cell carcinoma, and small-cell lung carcinoma which characteristically shows chromosome 3p14-3pter deletions [85]. Additionally, (t(3;8)(p25;q21)) translocations affecting the RAF-1 gene locus were detected in mixed parotid gland tumors [86, 87]. The human *c-raf-2* pseudogene is located on the tip of the short arm of chromosome 4 [81]. This region contains several polymorphic restriction enzyme sites which made it a useful marker for a genetic determinant of Huntington's chorea, as genes responsible for this disease are correlated with this region [88].

The murine *c-raf-1* homolog maps to chromosome 6 [89, 90]. RFLP analysis showed that murine *c-raf-1* is located approximately 16 centimorgans from the mouse immunoglobulin-kappa light-chain gene [91]. Several structural and numerical alterations of this chromosomal region have been reported in granulocytic leukemias [92].

The *C. elegans raf* homolog was mapped between the *unc 44* and *deb-1* genes on chromosome IV [79].

In *D. melanogaster* the *D-raf-1* gene was mapped by *in situ* hybridization on salivary gland chromosomes. *D-raf-1* is located in the 2F5-6 region near the tip of the X-chromosome [77], whereas *D-raf-2*, a raf-related gene, is located on chromosome 2 at position 42A2-5 [78].

7.4.2 *A-raf*

In humans, *A-raf* maps to the X-chromosome at position Xp11.2. This region belongs to an evolutionary conserved linkage group composed of *A-raf*, *synapsin I* (*syn*), *TIMP* and *properdin* [93], and it is of interest in a variety of human diseases including Duchènne muscular dystrophy [94], Menkes syndrome [95], and testicular feminization [96]. Both 3'ends of the human *A-raf* gene and of the *syn* gene which encodes a neuronal-specific phosphoprotein are suggested to be involved in neuronal diseases, as they share the same sequences oriented in opposite directions. A second locus on chromosome 7 at 7p11.4-7q21 contains the pseudogene *A-raf-2*.

In the murine genome, *A-raf* maps on the X-chromosome 10–17 centimorgans proximal to the hypoxanthin phosphoribosyl transferase gene (HPRT) [97, 98].

7.4.3 *B-raf*

Human *B-raf* is located on chromosome 7q34 [73, 74]. This places *B-raf* in an area which is involved in malignancies resulting from either chromosomal deletions or translocations [99]. For example, such events affecting sequences within 7q22–7q34 have been observed in glioma [99] and leiomyoma (del 7q22–q32) [100]. This chromosomal region may be susceptible to such alterations owing to the proximity of sequences which are prone to breakage under certain conditions. Such fragile site-loci are found on either side of the *B-raf* gene at 7q32.2 (FRA7H) and 7q36 (FRA17). The human *B-raf* pseudogene maps to chromosome Xq13.

7.5 Tissue distribution of Raf

7.5.1 *C-raf-1*

In man, *c-raf-1* is expressed as a 3.4 kb transcript while in mouse, the transcript is 3.1 kb in length. Thirty-six different murine tissues from adult and fetal animals have so far been examined for *c-raf-1* expression. Transcripts were found in all tissues, with highest levels in striated muscle, cerebellum and fetal brain, and lowest levels in skin, small intestine, thyroid and pancreas [54]. In mouse testis, *c-raf-1* mRNA has been detected in germ cells from type A and B spermatogonia through the round spermatid stage, with highest levels observed in pachytene spermatocytes, but was not found in residual bodies [101]. In addition to tissues, an assortment of murine cell lines was also tested for *c-raf-1* expression. Low expression levels were seen in Wehi cells, growth factor-dependent myeloid FDC-P1, and NFS-60 cells. Highest transcription levels were observed in several tumor cell lines such as EL-4, HCM 1416 and 1417 cells (mouse T-cell lymphoma lines [38, 47]), and in a mouse pancreatic epithelial tumor cell line [46]. Interestingly, *c-raf-1* expression is also increased in chemically induced lung tumors [13]. Transcriptional control elements of the *c-raf-1* gene remain to be elucidated.

In chicken, *c-mil* encodes two mRNA species generated by alternative splicing [102]. These two transcripts differ at least by the absence or presence of the 60 nucleotide exon E7a (Fig. 7.2). Analysis of the expression pattern revealed that mRNA lacking E7a (type 6C) is present in all tissues, whereas mRNA containing E7a (type 1A) was detected only in skeletal muscle, heart and brain [103]. The ubiquitous expression of type 6C mRNA suggests a general role for the 71 kDa protein, whereas the restricted expression pattern of type 1A mRNA indicates a tissue-specific function of this Raf isoform.

In *Xenopus*, a 3.1 kb *raf* mRNA is present at low levels in adult tissues including skin, testis, stomach and intestine, and at high levels in oocytes from stage I to VI. *Raf* mRNA in unfertilized eggs seems to be maternally expressed and levels decrease upon fertilization [58]. In *Drosophila*, *D-raf* is expressed as a single mRNA of 2.9 kb. Northern blot analysis of RNA from various developmental stages showed that the amount of D-raf mRNA is relatively high during the first 4 hours of embryonic development, whereas levels of expression are lower through the remaining developmental stages [77]. Abundance of the *D-raf* gene transcript in the adult ovary suggests transfer of the maternal mRNA into the ooplasm; in fact, the transcripts accumulate in unfertilized eggs.

7.5.2 *A-raf*

Expression of *A-raf* has been examined in whole-mouse embryos, several adult tissues and in murine and human cell lines. The *A-raf* mRNA is 2.6 kb in length, in rodents as well as in humans. In contrast to *c-raf-1*, *A-raf* shows high specifity in its tissue distribution [54]. *A-raf* mRNA is mainly found in mouse epididymis and ovary and to a lesser extent in testis and kidney, with expression levels varying 100-fold between these tissues. In testis, *A-raf* is expressed predominantly in the somatic compartment (Leydig cells) as two transcripts. In addition to the 2.6 kb mRNA,a rare 4.3 kb transcript was detected [69]. The localization of *A-raf* transcripts in steroid hormone responsive tissues may be a consequence of GRE/PRE hormone response elements found in the 5' region of the *A-raf*-gene [66].

7.5.3 *B-raf*

B-raf is expressed most abundantly in neuronal tissues with highest levels in the cerebrum. The sizes of these somatic transcripts are 10 and 13 kb, respectively. In addition to low levels of the somatic transcripts, mouse testis also contain two specific transcripts of 2.6 and 4.0 kb [54, 69]. *B-raf* is the only raf gene in mammals that undergoes alternative splicing. Transcripts in all tissues differ in the region between exon 8 and 9, where some contain a stretch of 36 bp (exon 8b) coding for additional 12 amino acids located downstream of CR2. In others, alternative splicing of exon 10 has been reported which results in the presence or absence of 40 amino acids in the region upstream of CR3, affecting the hinge region between the regulatory and catalytic domain. This modification is restricted to neuronal tissues and heart (J. V. Barnier, personal communication).

7.6 Protein structure

All three Raf isoforms are cytosolic phosphoproteins with molecular weights of 72–74 kDa (human c-Raf; 648 aa [53]), 70–72 kDa (human A-raf; 606 aa [65]) and 90–95 kDa (human B-raf; 765 aa [71, 72]). Variations in molecular weight are due to different phosphorylation states. Raf proteins share a common architecture with three conserved regions (CRs) embedded in variable sequences (numbers in text refer to human Raf-1; Fig. 7.3). CR1 (residues 62–192) contains a Zn-finger motif of the type $CX_2CX_9CX_2C$ which is presumably buried within the molecule in order to stabilize the hydrophobic residues while the Raf kinase is present in the cytosol. Upon membrane association of Raf, this domain is suggested to interact with the lipid bilayer [104]. CR1 and CR2 represent the autoinhibitory domain of the enzyme. CR1 also contains the

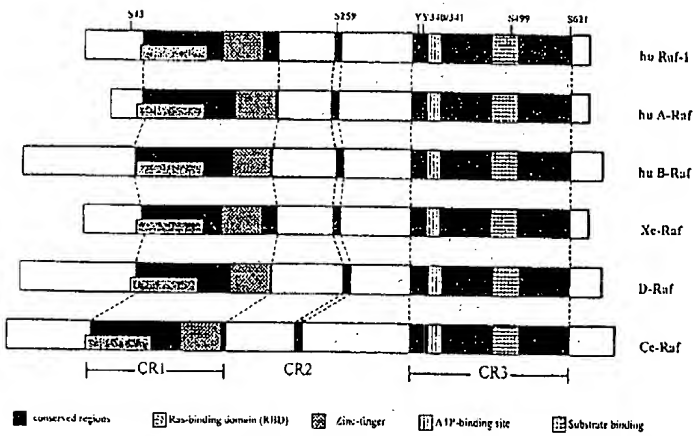

Figure 7.3 Structure of Raf kinases. Hu, human; Xe, *Xenopus*; D, *Drosophila*; Ce, *Caenorhabditis elegans*; CR1 (residues 62–196; numbers refer to human Raf-1), CR2 (255–268) and CR3 (331–625) represent conserved regions. Phosphorylation sites given for Raf-1 are not necessarily present in the other Raf kinases.

specific binding site (RBD, Ras binding domain) for the Raf regulator Ras [105]. CR2 (residues 254–268) is rich in serine and threonine residues, one of which is a major phosphorylation site *in vivo*. CR3 (residues 331–625) represents the kinase domain and is located in the carboxy-terminal half of the protein (Fig. 7.3).

The catalytic domain of Raf contains all XI kinase subdomains (see Chapter 1) and shows homology of approximately 30 % to other Ser/Thr protein kinases including Mos [106], protein kinase C [107, 108], cGMP-dependent kinases [109] as well as to tyrosine-specific protein kinases including members of the Src family and receptor protein tyrosine kinases [110–113]. Interestingly, a serine residue is found in Raf-1 (Ser499), but not in A- or B-Raf, at a position corresponding to the major activatory autophosphorylation site of Src (Tyr416) in subdomain VII [114]. Homologies of CR3 within the Raf kinase family are more than 75 %. The function of the conserved regions was highlighted by various types of deletion mutants. Dominant-negative mutants consist of either CR1 alone or CR1 and CR2, and thus, miss the CR3 region [115], whereas transforming mutants have lost the regulatory CR1 and/or CR2 and are constitutively active. The minimal transforming sequence corresponds to the intact CR3 domain [116]. Furthermore, N-terminal truncations [17, 19, 117, 118], alteration of the Zn-finger motif, as well as any kinase-activating mutation (e.g. Tyr340→Asp and Tyr341→Asp) lead to oncogenic versions of the enzyme [118–120]. Point mutations in the CR3 domain were described in a mouse model for chemically induced lung carcinoma and lymphoma. The predominant mutation was the exchange of amino acid 533 (Ser→Phe). All observed mutations cluster in a small region that might form the substrate pocket. This was suggested by computer modeling of the structure of Raf-1 using

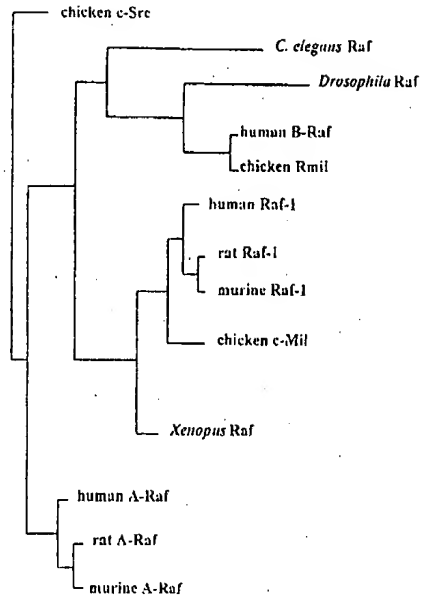

Figure 7.4 Phylogenetic analysis of Raf protein sequences. The tree was generated using the Phylogenetic Analysis Using Parsimony program developed by Swofford and represents an average obtained by boot strap analysis.

the coordinates for cyclic AMP-dependent protein kinase (see Chapter 2). As compared with wild-type Raf-1 these mutants exhibited an elevated kinase activity following triple co-expression with Ras and the tyrosine kinase Lck in insect cells. An explanation for this observation lies in altered interaction of CR3 with a putative inhibitor, or the regulatory N-terminal half of the Raf molecule [13].

The conserved regions of Raf kinases are separated by variable stretches which differ between the three Raf isoforms, but are highly conserved between different vertebrate species. An alignment of primary sequences of Raf from *C. elegans* (Ce-Raf; 813 aa; 90 kDa; [79, 121]), *Drosophila* (D-Raf; 782 aa; 75 kDa; [78]), *Xenopus* (Xe-Raf; 639 aa; 72 kDa; [57, 58]) and human (c-Raf; 648 aa; 72 kDa; [53]) reveals an overall identity of 38 % between *C. elegans* and human Raf, 46 % between *Drosophila* and human, and 34 % between *C. elegans* and *Drosophila* Raf. Analysis of the relationships between members of the Raf protein sequences indicates that B-Raf is more closely related to the non-vertebrate Raf proteins than it is to Raf-1 and A-Raf (Fig. 7.4).

In the plant *Arabidopsis*, a protein (CTR1) involved in the ethylene signaling pathway was identified as a member of the Raf kinase family [80]. Significant homology (41 % when compared with human Raf-1) was found in the carboxy-terminal half of the protein that corresponds to the catalytic domain. As in human Raf-kinases, a Zn-finger motif was identified in the N-terminal region of the protein; however, with unusual spacing of the cysteine residues. Thus, it is not likely that CTR1 binds to the

same effector molecules as Raf-1. Other interesting features of the N terminus of CTR1 include an unusually, high content of glycine and serine/theronine residues that was also reported for the N terminus of B-Raf [72].

7.7 Raf-1: role and function in signal transduction

7.7.1 Raf-1 and the cytoplasmic kinase cascade

The transmission of extracellular signals to intracellular target sites is achieved by a network of interacting proteins and leads to distinct physiological responses. Among intracellular signaling pathways, the Raf-MEK-MAPK-dependent signaling pathway is of special interest since its deregulation results in oncogenic transformation. The pathway is activated by binding of a growth factor to its receptor on the cell surface (see Chapter 9). As shown in Fig. 7.5, Raf is at the helm of a kinase cascade consisting of the MAPK-activating kinase (MEK) and MAP kinase (MAPK).[1] MAPK has a broad range of substrates including nuclear regulatory proteins. This cascade provides a link between receptor activation and phosphorylation-induced changes in gene expression. Not only the genes, but also the functional hierarchy of the cascade is highly conserved in evolution as analogs of all its components are present in different species including yeast [122, 123], *C. elegans* [124, 125], *Drosophila* [126] and mammals [104] (see also Chapter 9). As expected, the cytoplasmic kinase cascade is tightly controlled, and there are feedback phosphorylation of unknown significance as well as cross-regulations between different cascades [127]. We will focus on the control of Raf-mediated signaling that involves the small GTPase proto-oncogene Ras.

7.7.2 Regulation of Raf function

7.7.2.1 Ras connects Raf to the kinase cascade

The best known regulator of Raf activity is Ras [104, 105]. The conversion of GDP-bound Ras to the GTP-bound form is catalyzed by nucleotide exchange factors. Upon activation, tyrosine kinase receptors recruit the GDP/GTP exchanger Sos with the aid of adapter proteins such as Grb2 or Grb2/Shc [128]. Only GTP-Ras is able to bind and activate effector molecules such as Raf and phosphatidylinositol-3-OH-3-kinase [105, 129]. The activation of Raf-1 involves a physical interaction between Raf-1 and Ras. This requires a highly conserved amino acid domain (RBD, Ras-binding domain) located in CR1 [130, 131]. Experiments using site-directed mutagenesis showed that a mutation in this region (Arg89→Leu) was sufficient to abolish the Ras-binding activity of the RBD [132]. Moreover, RBD-surrounding regions are also necessary for conformational integrity for Ras binding *in vivo*, because the Cys168 mutation which is not localized in the RBD, also affects the Raf/Ras interaction. Interestingly, the mutation in Cys168 activates Raf to an intermediate level, whereas the Arg89→Leu mutation does not increase kinase activity suggesting that the Zn-finger structure normally participates in negative autoregulation (J. T. Bruder and U. Rapp, unpublished).

[1] MAPK, mitogen activated protein kinase, alias ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK activating kinase

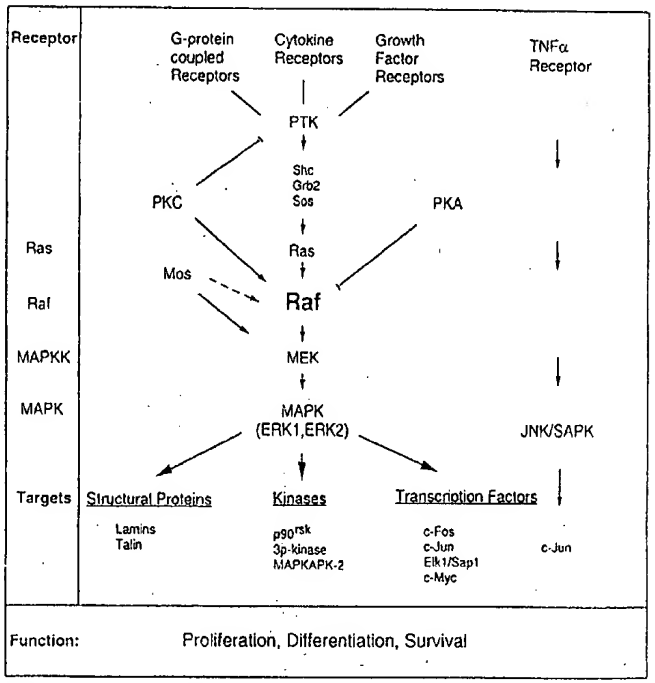

Figure 7.5 Raf-dependent signal transduction. Raf is activated upon stimulation of a variety of receptors and, together with MEK and MAPK, forms the conserved cytoplasmic kinase cascade. MAPK acts on various targets which finally determine important cellular functions. Arrows indicate activation, blocked lines inhibition. Dotted lines suggest activation. Putative connections between different signaling pathways are indicated. MEK, [mitogen activated protein kinase (MAPK)/extracellular regulated kinase (ERK)] kinase; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; JNK/SAPK, Jun-N-terminal/stress-activated protein kinases; TNF, tumor necrosis factor. For additional information see Fig. 9.2.

7.7.2.2 Modulators of Raf activation

The physical interaction between Ras and Raf alone is not sufficient to fully stimulate the kinase activity. Thus, activation of Raf in Ras-transformed cells is still growth factor-dependent [133]. Furthermore, co-expression of Raf-1 with oncogenic Ras in Sf9 insect cells failed to completely activate Raf-1 which was only achieved following

additional co-expression of v-Src [134, 135]. This suggests that, *in vivo*, the function of Ras is to position Raf-1 to the plasma membrane in the vicinity of a putative cofactor. Recent experiments in which Raf-1 was targeted to the membrane by fusing Raf-1 to the membrane-localization motif of the carboxy-terminal part of Ras, demonstrate that plasma membrane binding of Raf abrogates the requirement of Ras in the activation of the Raf-1 kinase [136, 137]. In order to find potential cofactors that participate in the activation of Raf, the yeast two-hybrid system was applied. So far, two members of the 14-3-3 protein family (see Chapter 3) were isolated that interact with the regulatory domain of Raf. They also interact weakly with CR3 [138–140]. The consequence of interaction between 14-3-3 proteins and Raf-1 appears to be a stabilization of the Ras/Raf complex, rather than a direct stimulation of Raf-1 kinase activity [141].

The activation of Raf-1 is not only regulated by Ras/Raf interaction. In addition, other G-proteins and protein kinases that directly phosphorylate Raf may regulate Raf kinase in a positive or negative way.

7.7.2.3 Protein kinase C – a positive regulator of Raf

Raf can be activated following treatment with the protein kinase C (PKC) activator 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [142–144]. The mechanisms by which various PKC isozymes regulate Raf-1 are not fully understood. For PKCα, it has been reported that it stimulates Raf kinase activity both, *in vivo* as well as *in vitro* via direct phosphorylation of Ser499 as shown in Fig. 7.6 [142]. Ser259, located in CR2 of Raf, is also required for optimal Raf-1 activation by PKCα. Additional PKC phosphorylation sites are present in Raf-1, but their functional significance remains to be elucidated.

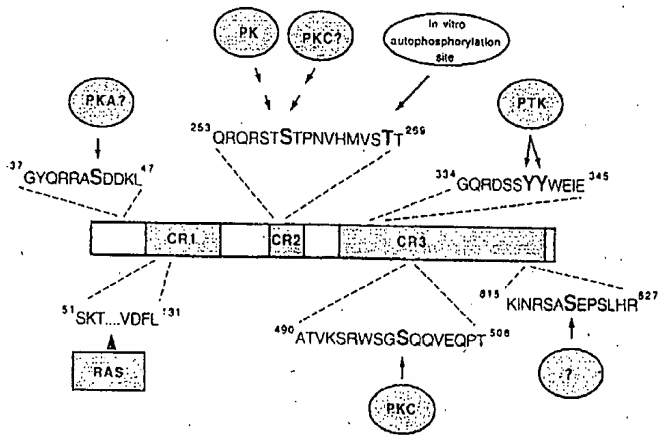

Figure 7.6 Location of Raf-1 phosphorylation sites and the Ras-binding domain (RBD). Phosphorylation sites are indicated by bold letters. PTK, protein tyrosine kinase; PKA, cyclic AMP dependent protein kinase; PKC, protein kinase C.

One member of the Raf kinase family, A-Raf, which lacks the serine at position 499 was also found to be phosphorylated *in vivo* following phorbol ester treatment. It will be important to determine which sites are involved.

7.7.2.4 cAMP-dependent protein kinase and Rap1a – negative regulators of Raf

A number of studies have implicated the activation of cyclic AMP (cAMP)-dependent protein kinase (cAPK, see Chapter 2) in the negative regulation of the Raf-MEK-MAPK cascade [145, 146]. Increases in cAMP levels correlate with the phosphorylation of the cAPK consensus sequence (RRXS) on Ser43 in CR1 [145] (Fig. 7.6). *In vitro*, cAPK directly phosphorylates Raf-1 as well as a synthetic peptide containing this consensus sequence. These observations suggest that inhibition of Raf-1 by cAMP is mediated by phosphorylation of Ser43 by cAPK that results in an attenuation of Ras-GTP binding. Interestingly, Ser43 is not located in the Ras-binding domain. To explain how its phosphorylation might affect Ras binding it was suggested that it creates a binding site in the N terminus for the Ras-binding domain, thus preventing Ras from binding.

Two other potential negative regulators of Raf-1 activity are cGMP-dependent protein kinase [147] and the cytoplasmic Ser/Thr kinase Pim-1 [148]. Both kinases share the cAPK consensus phosphorylation site. Whether these kinases can alter Raf-1 phosphorylation on Ser43 remains to be determined. In addition to direct phosphorylation, inhibition of Raf-1 may also be mediated by Ras-like GTPases such as Rap1a and Rap1b [105]. Since Rap1a interacts with Raf, as shown in the yeast two-hybrid system, and microinjection of Rap1a into cells antagonizes Ras-dependent activation of MAP kinase, a role for Rap1a in inhibition of Raf activation appears likely [105, 149].

7.7.2.5 Phosphorylation sites in the catalytic domain of Raf

In addition to regulation of Raf by PKC and cAPK, mutational analysis of phosphorylation sites in the catalytic domain suggests that tyrosine kinases also regulate Raf through direct phosphorylation. Two adjacent tyrosine residues (Tyr340 and Tyr341 were identified as phosphorylation sites of Raf-1 following co-expression with activated tyrosine kinases in Sf9 cells [120] (Fig. 7.6). Substitution of these tyrosines with alanines have a dominant-negative effect. Interestingly, introduction of negatively charged residues that mimic the effect of phosphorylation stimulates the basal activity of Raf-1. In addition, this Raf mutant is able to transform BALB/3T3 cells as observed for truncated versions of Raf-1 [150].

Another serine phosphorylation site located in CR3 was demonstrated to be important for kinase activity, Ser621 (Fig. 7.6). This residue is phosphorylated in starved cells at low levels, and its phosphorylation increased upon growth factor treatment. Substitution of Ser621 renders the kinase non-responsive to all activators [119]. Interestingly, Ser301 located near the kinase domain might be phosphorylated by MAP kinase along with several other sites in the N-terminal half of the molecule. This kinase has been shown to phosphorylate Raf-1 *in vitro* as well as *in vivo* [151, 152]. However, the function of this phosphorylation is not yet clear. We have speculated that the MAPK phosphorylation of Raf-1 may mediate the dissociation of active Raf-1 from the plasma membrane [104].

7.7.3 Downstream of Raf

7.7.3.1 MEK and MAP kinase

The MAPK activator MEK is the first substrate reported for Raf [153, 154]. MEK is a dual specificity protein kinase which becomes activated upon phosphorylation by Raf and phosphorylates a tyrosine and threonine residue in a TEY motif located in domain VIII of MAP kinases. The cloning of MEK cDNAs from mammalian cells [155], *Xenopus laevis* [156] and *D. melanogaster* [157] revealed a high degree of homology to the yeast genes byr1 and STE7 [158] which are involved in the mating pathway. Three isoforms of MEK have been described in mammalian, MEK1, -2, -3 [159, 160]. The activating phosphorylation sites on human MEK are Ser218 and Ser222 present in the catalytic domain [161]. These phosphorylation sites are highly conserved in all eukaryotic MEKs. Substitution of Ser218 or Ser222 by alanine completely prevented activation of MEK1 following mitogen-stimulation of cells [162]. A feedback phosphorylation on Thr292 of MEK by MAPK was observed *in vivo* [163]. A negative regulation of MEK via phosphorylation was suggested since *in vitro*, cdc2 kinase could phosphorylate MEK on sites Thr286 and Thr292, resulting in an inactivation of kinase activity [164].

Similar to Raf-1, MEK exhibits an extremely narrow substrate specificity with MAPK being its only substrate identified so far. The MAP kinase isoforms p44 (ERK1) and p42 (ERK2) belong to the family of proline directed protein kinases which share the common activatory phosphorylation site TXY. This motif is present in three subclasses (TEY, TPY, TGY). Each member of this family appears to be activated by a specific dual-specificity kinase involved in distinct signaling pathways [127].

In contrast to MEK and Raf, MAP kinases act on a variety of targets. Substrates include Ser/Thr kinases (p90 S6 kinase (RSK-2), MAPK-activated protein kinase-2, 3p-kinase), RNA-polymerase II, phospholipase A2, structural proteins (lamins, talins) and a number of transcription factors (c-Fos, c-Jun, c-Myc, Ets). Regulation of transcription factors by MAPK closes the gap between receptor-mediated events at the cell membrane and changes in gene expression in the nucleus (see Chapters 9 and 11). Although ERK1 and ERK2 are highly homologous and have many substrates in common, there is evidence that they differ in their substrate specificity, at least *in vitro* [165]. The consensus phosphorylation motif of the proline directed kinases is P/L-X-T/S-P, whereby the core sequence S/T-P is also recognized, however, with considerably lower affinity.

Although the integrity of the cytoplasmic kinase cascade Raf-MEK-MAPK has been confirmed in many systems, there is accumulating evidence that branch points exist. First, there might be additional substrates for Raf besides MEK. Second, additional MEK activators including MEKK (MEK kinase) [116] and c-Mos [167] have been reported. These observations suggest a role of MEK in more than one signaling pathway [168, 169]. Third, in addition to MEK, MAPK can apparently be activated by other kinases including the tyrosine kinase Lck [170] or by itself via an autophosphorylation event mediated by the transcription factor Elk-1, a member of the Ets family [171] (see also Chapter 11).

7.7.3.2 Targets of MAPK activity

Genes with Raf-1-responsive promoter elements include early (PEA-1, fos, egr-1) and late growth response genes CAD [172–175]. The serum response element (SRE) is a promoter element common to many cellular immediate-early gene promoters and is activated by growth factors as well as many oncogenes (Chapter 11). On the SRE of the *c-fos* gene, a complex between a serum response factor (SRF) and a ternary complex factor (TCF) is formed [176]. Mutational analysis of the *c-fos* SRE suggests that this complex is required for a full response to growth factor signals [177, 178]. The targets for the Raf-MEK-MAPK pathway in the *c-fos* promoter are the TCF proteins Elk-1 or Sap-1 [179] (see also Chapter 1). The activity of these Ets-family transcription factors is regulated by the phosphorylation of a cluster of C-terminal S/T-P motifs which follow the MAPK consensus sequence [180]. Experiments with both activated and dominant-negative mutants of MEK and ERK, show that MAPK activity is necessary for activation of Elk-1 and Sap-1 *in vivo* [181]. Additionally, *in vitro* analysis indicates that Elk-1 is a substrate for MAPK. Interestingly, Elk-1 protein seems to interact with MAPK and this might regulate kinase activity in a feedback manner [182].

The transcription factor Jun is another proto-oncogene that is regulated by the Raf-1 signaling cascade [183]. This transcription factor is functionally closely related to c-Fos and both nuclear proteins are part of the AP-1 complex (Chapter 11). The Jun-phosphorylation state seems to be a critical component in transcriptional activation. Smeal *et al.* demonstrated that Ras induces the phosphorylation of Ser63 and Ser73 within the transactivation domain of c-Jun, resulting in increased transactivation capacity [183]. Moreover, Pulverer *et al.* showed that purified preparations of MAPK also phosphorylate Jun at these sites [184]. Dominant-negative Raf-1 mutants block c-Jun phosphorylation in response to Ras, Src and ultraviolet light [183, 185]. The finding that dominant-negative Jun mutants block transformation by oncogenes activating the Raf-1 pathway indicates that Raf-1 induced phosphorylation and activation of Jun is necessary for NIH/3T3 cell-transformation [135].

Further experiments with c-Jun and stress-related cytokines led to the discovery of the JNK/SAPKs (Jun-N-terminal/stress-activated protein kinases [186]). These kinases represent another subfamily of proline-directed kinases distinct from the ERKs. They are regulated by extracellular signals including TNFα and IL-1. Agents that stimulate Jun phosphorylation such as ultraviolet light, strongly activate the JNK/SAPKs, but only weakly activate the ERKs [185, 186]. However, since JNKs seem to phosphorylate c-Jun at Ser63 and Ser73, the functional role of ERK-mediated Jun phosphorylation may be different. In fact, Minden *et al.* demonstrate that, unlike the JNKs, ERK1 and ERK2 do not phosphorylate the N-terminal part of c-Jun *in vitro*. Instead, they phosphorylate an inhibitory C-terminal site [187]. Similar observations have been made in our laboratory in the course of *in vitro* phosphorylation experiments (J. T. Bruder and U. Rapp, unpublished results). Additionally, activity of JNK/SAPKs, but not of ERKs correlate with the N-terminal phosphorylation of c-Jun *in vivo*. These findings suggest that two functionally distinct cascades, a MAPK- and a JNK/SAPK-dependent, are involved in the regulation of AP-1 activity.

In mammalian cells, at least two additional MAPK-related kinases appear to be regulated independently of the ERKs and JNKs: p88 which phosphorylates the fos tran-

scriptional activation domain [188] and p38 which seems to be the vertebrate homolog of the yeast kinase HOG1 [189]. Experiments are in progress to determine whether these kinases are also activated in a Raf-dependent manner.

In some instances, proteins of the Ets and AP-1 family have been found to act synergistically in transcriptional activation [190]. Since Bruder *et al.* showed that serum, TPA and Ras-induced expression from AP-1/Ets driven promoters requires Raf-1, it appears that Raf-1-induced MAPK activation is a common mechanism for transactivation through AP-1 and Ets binding sites [115, 135]. Additionally, Raf-1 activates expression through the NF-kB binding sites in the HIV-LTR which overlaps with an putative Ets-binding motif [191]. Ongoing experiments in our laboratory indicate that Raf-mediated activation of HIV-LTR-driven expression may also act through a Ets family transcription factor (E. Flory *et al.*, unpublished data).

7.8 Raf in the regulation of cellular processes

Initial work mainly focused on the role of Raf in cell transformation and proliferation. Later, studies in vertebrates and invertebrates revealed a crucial role for Raf in cell differentiation. It has now become obvious that Raf kinases are also involved in other cellular processes including proliferation, differentiation and survival [104, 135].

7.8.1 Proliferation and transformation

The first indication that Raf-1 plays a role in mitogenic processes came concomitantly with its identification as a viral oncogene [1]. Support for such a role evolved from studies with oncogenic forms of Raf which activated transcription [115, 172] and induced DNA synthesis upon microinjection into NIH/3T3 cells [192]. Furthermore, it has been reported that Raf kinases were activated in many cells upon treatment with growth factors. Raf-1 activation has been observed in many cell lines of fibroblastic and hemopoietic origin upon treatment with various stimuli including EGF, FGF, TPA, PDGF [193], GM-CSF [194], CSF-1 [195, 196], EPO [197] and an array of interleukins such as IL-2 [198], IL-3 [194], IL-4 and IL-6 [199]. Both, B-Raf and Raf-1 are activated in PC12 cells by stimuli which induce either proliferation (EGF, phorbol esters) or differentiation (NGF, FGF) [200, 201]. A-Raf becomes enzymatically activated following stimulation of cells with PDGF, EGF, FGF, NGF, and TPA (S. Grugel and U. Rapp, unpublished data). However, we do not know any factor that specifically activates only one member of the Raf kinase family.

Using *c-raf-1* antisense constructs and dominant-negative Raf-1 mutants, Kolch *et al.* have shown that Raf-1 is essential for mitogen-induced proliferation and oncogene-dependent transformation of NIH/3T3 cells [202]. However, although Raf is required for mitogenic responses, oncogenic Raf is not sufficient to achieve growth factor independence. Lowered growth factor requirement was observed in v-Raf expressing NIH/3T3 [192] and IL-3-dependent 32D cells [203]. Experiments in the latter cell system showed that co-expression with *v-myc* complements *v-raf* and establishes growth factor (IL-3)-independent growth [203–205]. These findings point to at least two pathways

mediating proliferative response: a Raf-pathway and a Myc-pathway, one leading to Raf-1 activation and the other to *c-myc* induction [135, 206].

Recent reports demonstrate that the JAK/STAT pathway which is activated by many cytokine-receptors may also be involved in mediating proliferative responses [207, 208]. This pathway is Ras-independent and consists of two components that are members of the subfamily of cytoplasmic protein tyrosine kinases, termed the Janus kinases (JAKs) and their substrates, the transcription factors of the family of signal transducers and activators of transcription (STAT, see Chapter 8). Besides the Raf and the Myc pathway, this is a third major pathway described activated by receptors of the cytokines. There is accumulating evidence that there are cross-connections between the Raf, the JAK/STAT and may be the Myc pathway.

7.8.2 Cell differentiation and development

The Raf signal transduction pathway plays an important role in extracellular signal-regulated development. The first observation of a participation of Raf in differentiation processes was made in terminally differentiating erythroid cells [43]. The Raf signaling mechanism is shared by a wide variety of organisms for many different developmental processes. This includes ethylene response in *Arabidopsis* [80] and vulval development of *C. elegans*. In *Drosophila*, Raf kinase is involved in the determination of the terminal regions and the establishment of the dorsoventral polarity of the embryo, as well as in eye development. Raf is essential for the mesoderm induction in *Xenopus* blastocysts and for a diverse array of differentiation processes in mammalian cells. These observations show that the role of Raf in the development of organisms is highly conserved in evolution.

7.8.2.1 Caenorhabditis elegans

Dominant-negative forms of Raf in the nematode *C.elegans* (Ce-Raf) prevent vulval induction [79, 125]. This organism has proven to be a valuable model system for studying cellular signaling pathways. The development of the hermaphrodite vulva is one of the best characterized system regarding signaling events. The vulva of *C. elegans* is formed by specialized ectodermal cells that connect the gonad to the environment. During vulval development a signal from a gonadal anchor cell causes the underlying ectodermal precursor cells to generate vulval cells, whereas the other ectodermal cells with the same developmental potential generate non-specific epidermis. The Ras/Raf signal transduction pathway has been shown to be required for the determination of cells to vulval or epidermal cells. The inductive signal, an EGF-like protein encoded by *lin-3*, is expressed in the anchor cell. It activates the tyrosine-kinase-receptor let-23, a member of the EGF receptor subfamily. Activated let-23/EGFR transduces this signal in a linear cascade involving the Ras-homolog Let-60 and *sem-5* gene product. Sem-5 contains SH2 and SH3 domains (see Chapter 8) and acts like its mammalian homolog Grb-2 by linking the activated receptor to proteins in the Ras-complex. Let-60/Ras activates the Raf homolog Ce-Raf encoded by *lin-45*. Analogous to the vertebrate signaling cascade, a MAP-kinase homolog mediates the Ce-Raf effect in vulval develop-

ment. This enzyme was found independently by two groups and named Mpk-1 [125] and Sur-1 [124], respectively. It shows highest homology to rat ERK2. However, a Sur-1/Mpk-1-activating kinase (a MEK homolog) has not yet been identified, whereas genetic epistasis experiments led to the discovery of a downstream effector of Sur-1/Mpk-1, Lin-1. Its function, however, remains to be elucidated [124].

7.8.2.2 Drosophila

In *Drosophila*, several developmental processes rely on the Raf-dependent signaling cascade although different protein tyrosine kinase receptors are involved including Torso [209], Sevenless [210], EGF-receptor (DER) [211], and FGF-receptor (DFGF-R1) [212] (see also Chapter 9). For instance, Torso and Sevenless (Sev) determine the posterior structure of the embryo and the fate of the R7 precursor in eye development, respectively [213–215]; when activated, both tyrosine kinases initiate a signal transduction cascade that involves the same proteins including Drk (a SH2 adapter protein homolog of Grb2), Sos (a nucleotide exchange factor), Ras1, D-Raf, Dsor1 (a MEK homolog), and Rolled (a MAPK homolog). Based on genetic epistasis experiments, the functional order of these components has been identified [126, 157, 210, 214].

Activated by Torso, this cascade leads to the expression of Tailless and Huckebein determining head and tail differentiation, as a block in this cascade results in a non-segmented embryo without these structures. The activation of the Sevenless pathway promotes the differentiation of the R7 precursor into a photoreceptor in the ommatidium. Since constitutively active Ras or Raf could rescue dominant-negative mutants of Sev and Tor, and loss-of-function mutations of Raf block signaling from both the Torso and Sevenless receptor, it has been suggested that either Ras or Raf is sufficient to activate these pathways [126].

Another Raf-dependent pathway in the development of *Drosophila* is triggered by the EGF receptor homolog DER [211]. This plays a role in the arrangement of wing veins, in the regulation of eye development, and in the establishment of the dorsoventral polarity of the embryo [126]. Recently, it was shown that also the DFGF-R1 pathway uses the Raf-coupled signaling cascade [212]. The *Drosophila* FGF receptor homolog is required for the migration of tracheal cells and the posterior midline glial cells during embryonic development.

The fact that signaling induced by these four receptor tyrosine kinases overlap in the use of the Raf-coupled signaling pathway suggests that tyrosine kinase receptors and intracellular phosphotyrosine kinases are functionally equivalent in terms of their ability to activate the same intracellular signaling pathways [206, 216].

Future research will answer the question of how the specificity of developmental processes is achieved, considering the fact that these four receptor tyrosine kinases overlap in that they all use the Raf-dependent cytoplasmic kinase cascade.

7.8.2.3 Xenopus

Xenopus was the first vertebrate system studied where it was shown that Raf plays an important role in the early embryonic development. Raf participates in mesoderm induction and the development of posterior structures. Mesoderm induction is regulated

by two different mechanisms. Activin and transforming growth factor β induce the anterodorsal mesoderm, whereas basic fibroblast growth factor (bFGF) induces the posteroventral mesoderm. Injection of a dominant-negative Raf-1 mutant into animal cap explants completely blocked bFGF-stimulated mesoderm induction, whereas activin induction of mesoderm remained unaffected [217].

7.8.2.4 Mammals

Soon after the observation that Raf functions in proliferation, it became clear that it also participates in differentiating processes in mammals [43, 201, 218].

Raf was shown to mediate insulin-induced differentiation of 3T3 L1 cells into adipocytes [219]. Insulin treatment of 3T3 L1 cells results in a Ras-dependent phosphorylation of Raf-1 as well as MAPK and the 90-kDa S6 kinase (Rsk-2). There are two lines of evidence that Raf is essential for adipocytic differentiation. First, expression of oncogenic forms of Raf induces differentiation. Second, expression of a dominant-negative Raf mutant significantly blocks differentiation. Interestingly, in this system Raf does not act through phosphorylation of MAPK and Rsk-2, even though these kinases were induced by insulin treatment. This is shown by the fact that expression of *raf* oncogenes does not lead to MAPK or Rsk-2 activation, and that insulin-induced activation of these kinases is not blocked by dominant-negative Raf mutants. These findings indicate that insulin activates a Raf pathway and a Raf-independent MAPK-Rsk-2 pathway, of which the first is responsible for adipocytic differentiation [219]. Another system where Raf participates in differentiation processes are hemopoietic stem cells. Transformation of murine bone marrow cells with *v-raf* in combination with *v-myc* resulted in clonally related populations of mature B cells and mature macrophages, whereas transformation with either *v-raf* or *v-myc* alone led to transformed pre-B cells, and no mature B-cell or macrophage line was found [220, 221]. Furthermore, *v-raf* infection of B-lineage cells from Eμ-myc transgenic mice, where the immunoglobulin heavy chain enhancer (Eμ) forces expression of *c-myc*, can lead to a lineage switch from B-cells to macrophages. This demonstrates that dysregulation of Raf and Myc allows reprogramming of B-cells. The B-cell/macrophage switch might occur either by regression to a putative precursor or by direct adoption of the macrophage differentiation program [218]. These findings clearly indicate that combined expression of Raf and Myc influences the lineage determination in hemopoiesis [220].

Besides lymphoid and myeloid lineages, hemopoietic stem cells also generate the erythroid lineage. Infection of bone marrow cells with *v-raf* in the presence of suboptimal amounts of erythropoietin, efficiently produced colonies of well-differentiated hemoglobin-synthesizing erythroid cells. In this case, *v-raf* alone is sufficient for differentiation. On the other hand, cells infected by *v-raf* and *v-myc* did not undergo terminal differentiation, but proliferated at high rate. *V-myc* alone was unable to stimulate the formation of erythroid colonies [43]. In this system, it appears that Myc inhibits terminal differentiation, whereas the combination of Raf and Myc supports proliferation and differentiation up to but not including the terminal stage.

In addition to adipocytic and hemopoietic differentiation processes, Raf is also involved in neuronal differentiation. Treatment of rat pheochromocytoma cell line PC12 by nerve growth factor (NGF) leads to neurite outgrowth, whereas EGF treatment re-

sults in proliferation (see section 7.8.3). In these cells, NGF as well as EGF, FGF and TPA cause phosphorylation of Raf-1 and B-Raf [200]. The fact that oncogenic Raf substitutes for NGF regarding many effects, indicates that Raf kinases are principal mediators of NGF signaling leading to differentiation. It is possible that oncogenic forms of Raf-1 are mimicking the normal actions also of B-Raf, as it is B-Raf that is suggested to mediate NGF signaling [201, 222, 223].

7.8.3 Proliferation versus apoptosis versus differentiation – the role of Raf in cell fate determination

Findings from PC12 and hemopoietic cells indicate that the balance of the Raf and Myc pathway determines cell fates such as growth, apoptosis and differentiation (Fig. 7.7) [135]. Interestingly, in PC12 cells, EGF activates the Ras/Raf/MEK/MAPK pathway and promotes proliferation, whereas NGF induces differentiation into neuron-like cells using the same pathway [135, 201, 224, 225]. This raises the question as to the origin of the difference.

There are several lines of evidence suggesting that differences between these receptors are quantitative rather than qualitative. First, while NGF stimulation results in a persistent elevation of RasGTP, EGF produces only a short-lived rise in RasGTP [224, 226]. The same effect is seen for MEK [227] and ERK activation [224]. Second, constitutively active Ras, Raf or MEK, all permanently induce ERK activation and stimulate neuronal differentiation [222, 224, 228, 229]. Third, while stimulation of the endogenous EGF receptor (EGFR) does not lead to neurite outgrowth, stimulation of overexpressed EGFR has a differentiating effect. Similarly, a chimeric form of the human EGFR containing the cytoplasmic part of v-erbB also leads to differentiation upon EGF stimulation (U. Rapp and A. Ullrich, unpublished data). These findings indicate that prolonged activation of the pathway leads to differentiation, whereas short-lived activation is associated with proliferation [135, 225]. Additionally, we have evidence that the Myc pathway, together with the Raf pathway, is involved in cell fate determination in PC12 cells (U. Rapp, unpublished data).

Expression of inhibitory mutants of Myc alter the response of PC12 cells to EGF which then behaves like a differentiating factor. This suggests that Myc has an inhibitory effect on differentiation, although it is not dominant over the differentiation induced by *v-raf*, and that the response depends on the strength of the differentiation signal. Since the phenotypes of differentiated PC12 cells differ dependent on the Raf/

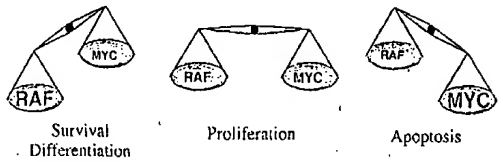

Figure 7.7 Model for Raf/Myc effects on cell fate. The Raf/Myc ratio determines whether the cell undergoes proliferation, differentiation or apoptosis.

Myc ratio, it is suggested that this ratio is instructive as to the type of differentiation that is induced [135] (U. Rapp, unpublished data). Myc does not inhibit neurit outgrowth in PC12 cells, nor is it inhibitory in the differentiation of pre-B to mature B-cells [221]. However, in erythroid and F9-cells, Myc is inhibitory in differentiation [43, 230]. This difference may be due to the fact that, in the case of PC12 and B-cells, differentiation is preceded by a round of proliferation, whereas in F9- and erythroid cells, differentiation is associated with growth arrest.

The Myc/Raf ratio also determines cell growth and apoptosis in 32D.3 cells [135]. This murine myeloid progenitor cell line is strictly dependent on IL-3 for survival and proliferation. Removal of IL-3 results in cell cycle arrest in G0–G1 followed by apoptosis (programmed cell death) [231]. In the presence of IL-3, expression of oncogenically activated Raf shortens G1 phase, thereby leading to an enhanced proliferation rate. Although *v-raf* is not sufficient for growth in the absence of IL-3, it has survival activity by suppressing apoptosis [203, 232]. In this respect, *v-raf* functions similarly to the Bcl-2 (B-cell lymphoma/leukemia-2) protein which also promotes survival of myeloid cells. Wang *et al.* showed that constitutively active Raf-1 acts synergistically with Bcl-2 in suppression of apoptosis [233]. The mechanism by which Raf-1 and Bcl-2 cooperate is not yet known. On the one hand, they appear to act through parallel pathways as Bcl-2 does not activate Raf kinase, and Raf-1 neither induces expression of endogenous *bcl-2* nor stimulates phosphorylation of the Bcl-2 protein [233]. On the other hand, Bcl-2 was found associated with the C-terminal half of Raf-1. Considering the distinct cellular distribution of Bcl-2 which is found in the outer mitochondrial membrane and nuclear envelope, it is imaginable that Bcl-2 guides Raf-1 to these cell compartments and thereby to substrates whose phosphorylation is critical for survival [233, 234]. A small G-protein presumably participates in the process as R-Ras was found to bind both Raf-1 and Bcl-2. Other observations indicate the participation of *v-myc* in proliferation and apoptosis. While co-expression of *v-myc* and *v-raf* leads to proliferation and abrogation of IL-3 dependence [232], expression of *v-myc* alone accelerates apoptosis of 32D.3 cells in the absence of IL-3 [231].

These findings form the basis for a model that both the Raf and the Myc pathway are required in cell fate determination. Dependent on the ratio of Raf and Myc, the cells undergo apoptosis, proliferation or differentiation (Fig. 7.7).

7.9 Future perspectives

A physiological role of Raf kinases has been established in processes leading to long-term changes such as cell cycle progression, suppression of apoptosis and induction of differentiation. Major questions remain regarding the functions of Raf in these processes.

The mechanism by which Raf exerts its function in cell cycle progression is not well understood. There are at least two steps in the cell cycle where Raf is required, in G0/G1 transition [235] and G1 progression [203, 232]. In addition Raf may also play a role in G2/M transition [235]. The recent observation of a physical interaction between Raf and the phosphatase Cdc25A (see Chapter 6) is the first hint for a direct link between Raf and the cell cycle (D. Beach, personal communication).

With regard to the relevance of Raf kinases in therapy of human tumors, it has been reported that the Raf oncogene relates to radiation resistance [236, 237] whereas expression of *c-raf-1* protooncogene correlates with radiation sensitivity [238]. Although present data do not allow a definitive evaluation, it seems reasonable to speculate that the anti-apoptotic activity of activated Raf may form a basis for altered radiation sensitivity. Elucidation of the association of Raf with radiation sensitivity may help to evaluate cancer therapies.

So far, the role of Raf in mammalian differentiation processes has been studied predominantly in cell culture. Recent experiments in transgenic mice indicate that Raf activation is critical for embryogenesis as both, dominant-negative and constitutive active versions of Raf-1 caused lethality (T. Beck and U. Rapp, unpublished data). The use of the embryonic stem cell system and knock-out techniques will be helpful to evaluate the role of Raf in early embryonic development.

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8 Non-receptor protein tyrosine kinases

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8.1 Introduction

Protein tyrosine kinases are important components of numerous, diverse, signal transduction pathways. Such pathways are conserved within cells from organisms as simple as sponge to those as complex as mammals. Signal transduction itself is the process whereby an extracellular signal is conveyed to the central organizing body of the cell, the nucleus. Within the nucleus the signal is delivered to the transcriptional machinery, where it is converted into a physical response. Such signals commonly result in cellular division, differentiation, alterations in cell shape and/or mobility or induction of expression of a novel set of proteins (for a review, see [1]). By corollary, loss of control of these pathways could potentially lead to a state of constant signaling resulting in uncontrolled cell growth, a condition known as cancer.

Protein tyrosine kinases have been divided into two groups, the receptor class and the non-receptor class. The receptor class is composed of a large family of cell-surface proteins, which as their name suggests act as receptors for a variety of ligands (see Chapter 9). They are transmembrane proteins, having both a domain which is extracytoplasmic as well as a cytoplasmic domain. They are in turn subclassified into a number of more closely related families [2]. The second class of tyrosine kinases are known as the non-receptor type as they have no extracellular sequences and do not span the plasma membrane. These too, are subclassified into a number of families which are quite diverese in their localization and expression. To date there are seven subdivisions

Table 8.1 Expression and subcellular localization patterns of non-receptor tyrosine kinase families

Family	No. of members	Expression pattern	Sucellular localization
JAK	3	Mostly hematopoietic tissues	Cytoplasmic
SYK	2	SYK in B cells and platelets ZAP-70 in T cells	Cytoplasmic
Abl	2	Ubiquitously expressed	Nuclear and cytoplasmic
Src	9	Ubiquitous and specific (see Table 8.2)	All membrane
Csk	1	Ubiquitously expressed	Cytoplasmic
FAK	1	Ubiquitously expressed	Cell adhesion plaques
Fps	3	Fer, Flk widely expressed Fps mostly hematopoicitic cells	Nuclear and cytoplasmic Cytoplasmic
Itk	3	Some hematopoietic cells, Tec is in liver also	Cytoplasmic

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